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(54) Title: SCREENING METHODS FOR AGENTS THAT MODULATE DEGENERATIVE DISORDERS ASSOCIATED WITH BCL-2-AND BIM

(57) **Abstract:** The present invention discloses methods of screening for agents that modulate the level and/or functional activity of a pro-apoptotic protein or that modulate the level and/or functional activity of a pro-survival protein for treating or preventing degenerative disorders. Also disclosed is a genetically modified non-human animal model for use in these methods, which has a partial or complete loss of function in at least one allele of a pro-apoptotic gene, especially a *bim gene*, and a partial or complete loss of function in at least one allele of a pro-survival gene, especially a *bcl-2* gene.

SCREENING METHODS FOR AGENTS THAT MODULATE DEGENERATIVE DISORDERS ASSOCIATED WITH *bcl-2 and bim.*

FIELD OF THE INVENTION

THIS INVENTION relates generally to degenerative disorders. More particularly, the present invention relates to conditions associated with the activation or inactivation of apoptosis, including degenerative disorders characterised by inappropriate cell death and/or inappropriate cell proliferation. Even more particularly, the present invention relates to the use of agents that modulate the level and/or functional activity of a pro–apoptotic protein, especially a BH3–only protein, or that modulate the level and/or functional activity of an anti–apoptotic or pro-survival protein, for treating or preventing degenerative disorders. The present invention also relates to methods of screening for agents that modulate the level and/or functional activity of a pro–apoptotic protein or for agents that modulate the level and/or functional activity of a pro–survival protein, especially using a genetically modified non–human animal model having a partial or complete loss of function in at least one allele of a pro–apoptotic gene and a partial or complete loss of function in at least one allele of a pro-survival gene. Thus, the present invention also relates to such a model *per se* and more specifically to a genetically modified non–human animal model having altered *bcl–2* function and altered *bim* function. Even more specifically, the present invention relates to a genetically modified non–human animal which is homozygous or heterozygous for a disruption in the endogenous *bcl–2* gene and for a disruption in the endogenous *bim* gene. Accordingly, the present invention extends to the use of this animal model in screening not only for agents that modulate activation or inactivation of apoptosis but also for agents that modulate cell survival.

Bibliographic details of various publications referred to by author in this specification are collected at the end of the description.

BACKGROUND OF THE INVENTION

Apoptosis is a major biological process by which multicellular organisms eliminate superfluous, damaged or infected cells. It plays a critical role in embryonic development, tissue homeostasis and immune defence against pathogens. The Bcl–2 family of proteins (which comprises pro–and anti–apoptotic members) are key regulators of this

process. All Bcl–2 family members possess at least one of the four Bcl–2–Homology domains (BHI–BH4).

The *bcl–2* gene was first discovered as the proto–oncogene translocated to the immunoglobulin heavy chain locus in follicular B cell lymphoma. Unlike many other oncogenes which induce cell proliferation, Bcl–2 exhibits a death repressor activity. In normal cells, Bcl–2 is associated with intracellular membranes. Overexpression of Bcl–2 protects cells against a wide variety of physiological death signals and experimentally applied cytotoxic stimuli. Bcl–2 inhibits apoptosis by preventing activation of caspases, a family of asp–specific cystein proteases that elicit morphological features of apoptosis by cleaving vital cellular substrates.

Although the expression of Bcl–2 is widespread in immature tissues, mice in which the *bcl–2* gene has been disrupted are born normally. They then display pleiotropic abnormalities including growth retardation, smaller ears, atrophic thymus and spleen, hair hypopigmentation in the second hair follicle cycle, and the majority die between 2 and 6 weeks due to uraemia caused by polycystic kidney disease. Hematopoietic cells from these mice have abnormally increased sensitivity to many apoptotic stimuli.

The pro-apoptotic protein Bim was first cloned as a Bcl–2–interacting protein (O'Connor *et al.*, 1998). Its only similarity to any known protein is the short (9 amino acid) BH3 motif shared by all Bcl–2 family members. The BH3 region is required for its ability to bind Bcl–2 and for most of its cytotoxicity. In healthy cells, Bim is sequestered to the microtubule associated dynein motor complex *via* the dynein light chain (LC8; Puthalakath *et al.*, 1999). Certain apoptotic stimuli cause the release of Bim from this complex together with LC8 and can then translocate to and inactivate pro–survival Bcl–2–like molecules.

Bim plays a major non–redundant role in embryogenesis, in the control of hematopoietic cell death, and as a barrier against autoimmunity (Bouillet *et al.*, 1999). Sixty percent of $bim^{-/-}$ mice die during embryogenesis from a still unknown cause. $bim^{-/-}$ mice have a 2– to 5–fold elevation in blood leukocytes, especially B and T cells. The spleen and lymph nodes of young adult $bim^{-/-}$ mice contain 2– to 3– times more B and T lymphocytes than wild type (wt) littermates. In tissue culture, $bim^{-/-}$ lymphocytes are resistant to certain Bcl–2 inhibitable apoptotic stimuli, such as cytokine withdrawal and treatment with a calcium ionophore, but remain almost normally sensitive to other apoptotic stimuli, including glucocorticoids, γ–radiation, or PKC activation. These results

demonstrate that different death stimuli require different BH3–only proteins for apoptosis induction.

Lymphadenopathy progresses with age in *bim*$^{-/-}$ mice (on a mixed C57BL/6x129 genetic background) and 60% die before 1 year of age due to systemic lupus erythematosus (SLE)–like autoimmune glomerulonephritis. Importantly, a gene–dosage effect is observed in the *bim*$^{+/-}$ animals, as their characteristics are always intermediary between those of wild–type– and *bim*$^{-/-}$ animals.

SUMMARY OF THE INVENTION

In work leading up to the present invention, the genetic interaction between Bim and Bcl–2 was investigated by crossing *bcl–2*$^{+/-}$ mice and *bim*–deficient mice to produce double mutant mice. The inventors found unexpectedly that the loss of *bim* in *bcl–2*$^{-/-}$ mice almost completely abrogated the phenotypes caused by inactivation of *bcl–2*. In particular, it was shown that the *bcl–2*$^{-/-}$ *bim*$^{-/-}$ and even the *bcl–2*$^{-/-}$ *bim*$^{+/-}$ offspring developed into healthy adults with ears of normal length and normal body weight. It was also shown that the loss of a single allele of *bim* in *bcl–2*$^{-/-}$ mice fully restored normal kidney development, weight gain and health and ameliorated lymphopenia. Further, it was found that the loss of both *bim* alleles was required to reduce greying and prevent lymphopenia completely. These results demonstrate that Bim and Bcl–2 function as specific antagonists in several cell types, including the developing kidney, lymphocytes and melanocytes and that Bim levels set the threshold for initiation of apoptosis.

From the foregoing, the present inventors propose that certain BH3–only proteins could be significant targets for disease intervention. More particularly, it is proposed that disease progression in degenerative disorders characterised by inappropriate cell death, including those not caused by loss of a Bcl–2–like gene, can be halted by drugs that inhibit particular BH3–only proteins or their upstream regulators. Conversely, drugs that either up–regulate their activity or mimic their action on their pro–survival counterparts are proposed to have therapeutic potential in degenerative disorders characterised by inappropriate cell proliferation.

The present inventors have also reduced the different gene dosage effects of *bim* to practice in a genetically modified, non-human animal model which provides facile phenotypic screening for modulators of Bim and/or Bcl–2 function. In accordance with the present invention, genetically modified animals are provided each having a partial or complete loss of function in at least one allele of the endogenous *bcl–2* gene, which results in a reduced or abrogated level and/or functional activity of Bcl–2, and a partial or complete loss of function in at least one allele of the endogenous *bim* gene, which results in a reduced or abrogated level and/or functional activity of Bim. Advantageously, each of those animals (*e.g.*, *bcl–2*$^{-/-}$ *bim*$^{+/-}$, *bcl–2*$^{+/-}$ *bim*$^{+/-}$ or *bcl–2*$^{-/-}$ *bim*$^{-/-}$) has a predetermined phenotype which corresponds to a specific level and or functional activity of Bcl–2 and a specific level and or functional activity of Bim. Thus, a candidate agent may be

conveniently screened for its ability to modulate apoptosis or cell survival by administering the candidate agent to a genetically modified animal of the invention and detecting a change in phenotype of the animal. The detection of a predetermined phenotype associated with a different level and/or functional activity of a molecule selected from Bim or Bcl–2 or other pro-survival agent, relative to the level and/or functional activity of that molecule in the absence of administering the candidate agent to the animal, is indicative of the candidate agent being a modulator of apoptosis or cell survival.

Modulators obtained using these genetically modified animals will be useful *inter alia* for treating and/or preventing conditions associated with the activation or inactivation of apoptosis, including degenerative disorders characterised by inappropriate cell proliferation or inappropriate cell death, respectively. Degenerative disorders characterised by inappropriate cell proliferation include, for example, inflammatory conditions such as inflammation arising from acute tissue injury including, for example, acute lung injury, cancer including lymphomas, such as prostate hyperplasia, genotypic tumours, autoimmune disorders, tissue hypertrophy etc. Degenerative disorders characterised by inappropriate cell death include, for example, acquired immunodeficiency disease (AIDS), kidney disorders including polycystic kidney disease, cell death due to radiation therapy or chemotherapy, neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, etc.

Accordingly, in one aspect of the present invention, there is provided a method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation or inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent which modulates the level and/or functional activity of a BH3–only protein for a time and under conditions sufficient to treat or prevent the disorder.

In another aspect, the present invention contemplates a method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation of apoptosis, the method comprising administering to the patient an effective amount of an agent which reduces, abrogates or otherwise suppresses the level and/or functional activity of a BH3–only protein for a time and under conditions sufficient to treat or prevent the disorder.

In yet another aspect, the present invention encompasses a method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the

inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent which increases, enhances or otherwise elevates the level and/or functional activity of a BH3–only protein for a time and under conditions sufficient to treat or prevent the disorder.

Suitably, the BH3–only protein is selected from BAD, BID, Hrk/DP5, Bik/Nbk, Blk, Bmf, NOXA, PUMA or Bim, or variants thereof. In a preferred embodiment, the BH3–only protein is Bim.

According to another aspect of the present invention, there is provided a non–human genetically modified animal having a partial or complete loss of function in one or both alleles of the endogenous *bcl–2* gene and in one or both alleles of the endogenous *bim* gene. The genetically modified animal preferably has an altered *bim* gene and an altered *bcl–2* gene. Suitably, the genetically modified animal is homozygous or heterozygous for one or both of the alterations. In a preferred embodiment of this type, the genetically modified animal comprises a disruption in at least one allele of the endogenous *bim* gene and in at least one allele of the endogenous *bcl–2* gene. Preferably, an individual disruption has been introduced into the genome of the animal by homologous recombination with a targeting construct in an embryonic stem cell such that the targeting construct is stably integrated in the genome of the animal, and wherein the disruption results in a reduced or abrogated level and/or functional activity of an expression product corresponding to the at least one allele.

Suitably, the genetically modified animal is selected from the order Rodentia. In a preferred embodiment, the non–human animal is a mouse.

In another aspect, the invention embraces a cell having a partial or complete loss of function in one or both alleles of the endogenous *bcl–2* gene and in one or both alleles of the endogenous *bim* gene.

In accordance with the present invention, the genetically modified animal and cells derived therefrom provide a facile means for phenotypically screening biologically active agents that modulate Bim and/or Bcl–2 function. These screening methods are of particular use for determining the specificity and action of drugs that may interact with Bim and/or Bcl–2. The genetically modified animal is also useful as a model to investigate the role of Bim and/or Bcl–2 in the activation or inactivation of apoptosis in several cell types, including the developing kidney, lymphocytes, melanocytes and neural cells.

Thus, in another aspect of the present invention, there is provided a method for screening a candidate agent for the ability to modulate apoptosis or cell survival, comprising:

(a) administering a candidate agent to a genetically modified non-human animal having a partial or complete loss of function in at least one allele of the endogenous *bcl–2* gene, which results in a reduced or abrogated level and/or functional activity of Bcl–2, and a partial or complete loss of function in at least one allele of the endogenous *bim* gene, which results in a reduced or abrogated level and/or functional activity of Bim, wherein the genetically modified animal has at least one predetermined first phenotype in the absence of administering the candidate agent to the animal, and

(b) detecting at least one predetermined second phenotype that correlates with a different level and/or functional activity of a molecule selected from Bim, Bcl–2 or other Bim-interacting pro-survival Bcl–2 family member, than the level and/or functional activity of the molecule that correlates with the or each predetermined first phenotype, which indicates that the candidate agent is a modulator of apoptosis or cell survival.

In a preferred embodiment, a predetermined first phenotype correlates with a different copy number of a gene selected from the endogenous *bcl*-2 gene and the endogenous *bim* gene, than the copy number of that gene which correlates with the predetermined second phenotype.

Suitably, the other Bcl–2 family member is selected from Bcl–xL, Mcl–1, A–1 and Bcl–w. Preferably, the other Bcl–2 family member is Bcl–xL.

In yet another aspect, the invention provides an agent which modulates apoptosis or cell survival, wherein the agent is obtained by the method as broadly described above.

In still yet another aspect, the invention encompasses a composition for modulating apoptosis or cell survival, comprising an agent as broadly described above, together with a pharmaceutically acceptable carrier.

In a further aspect, the invention contemplates a method for modulating the activation or inactivation of apoptosis in a cell, the method comprising administering to a patient in need of such treatment an agent as broadly described above for a time and under conditions sufficient to modulate the activation or inactivation in the cell.

According to another aspect of the invention, there is provided a method for treatment and/or prophylaxis in a patient of a condition associated with the activation or inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent identified by the screening method broadly described above for a time and under conditions sufficient to treat or prevent the condition.

In yet another aspect, the invention encompasses a method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation or inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent which modulates the level and/or functional activity of Bim and which has been identified by the screening method broadly described above for a time and under conditions sufficient to treat or prevent the disorder.

In another aspect, the present invention contemplates a method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation of apoptosis, the method comprising administering to the patient an effective amount of an agent which reduces, abrogates or otherwise suppresses the level and/or functional activity of Bim and which has been identified by the screening method broadly described above for a time and under conditions sufficient to treat or prevent the disorder.

In yet another aspect, the present invention contemplates a method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent which increases, enhances or otherwise elevates the level and/or functional activity of Bim and which has been identified by the screening method broadly described above for a time and under conditions sufficient to treat or prevent the disorder.

The invention also encompasses the use of the genetically modified animals and agents as broadly described above in the study of apoptosis and cell survival and in the study of degenerative disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photographic and graphical representation showing that loss of a single *bim* allele prevents growth retardation and premature death of Bcl–2–deficient mice. **a,** wt and *bcl–2*$^{-/-}$ mice from the same litter at 4 weeks. **b,** *bcl–2*$^{-/-}$ *bim*$^{+/-}$, *bcl–2*$^{+/+}$ *bim*$^{-/-}$, *bcl–2*$^{-/-}$ *bim*$^{-/-}$ mice on the C57BL/6 background at 8 weeks. *bcl–2*$^{-/-}$ *bim*$^{+/-}$ have a normal size and normal ears, but still turn grey at the second hair follicle cycle. Hair colour change is prevented in *bcl–2*$^{-/-}$ *bim*$^{-/-}$ mice. **c,** Mean weight of *bcl–2*$^{-/-}$ *bim*$^{+/+}$, *bcl–2*$^{-/-}$ *bim*$^{+/-}$, *bcl–2*$^{-/-}$ *bim*$^{-/-}$ and *bcl–2*$^{+/-}$ *bim*$^{+/-}$ mice on the C57BL/6 background. Note that most *bcl–2*$^{-/-}$ mice have died by 6 weeks of age.

Figure 2 is photographic representation showing that polycystic kidney disease of *bcl–2*$^{-/-}$ mice is prevented by absence of a single *bim* allele. **a** to **c,** Kidney sections (x 10) contrasting the abundant large thin–walled cysts in a typical *bcl–2*$^{-/-}$ mouse (b) with the normal histological appearance and complete absence of cysts in a *bcl–2*$^{-/-}$ *bim*$^{+/-}$ mouse (c) and a wt mouse (a). **d** and **e,** Sections (x 5) through the metanephros of E15.5 wt (d) and *bcl–2*$^{-/-}$ (e) embryos, showing the first signs of abnormal development in the *bcl–2*$^{-/-}$ kidney – a reduction in size and a narrower nephrogenic zone (nz) in the mesenchymal area. **f,** Section (x 10) through an E13.5 metanephros from a heterozygous *bim–βgal*$^{+/-}$ embryo, showing strong β–galactosidase (blue) staining in the mesenchymal cap area. Sections in **a–e** were stained with hematoxylin and eosin.

Figure 3 is photographic representation showing that greying elicited by Bcl–2 deficiency reflects Bim–driven melanocyte death. **a,** Skin section (x 15) of a *bcl–2*$^{-/-}$ *bim*$^{-/-}$ mouse during the anagen (growth) phase of the second hair follicle cycle, showing hair follicles with melanin (m) in the shafts and melanocytes (mc) in the bulb. **b,** Skin section (x 15) of a *bcl–2*$^{-/-}$ *bim*$^{+/-}$ mouse during the anagen phase, showing hair follicles lacking melanin and melanocytes. **c,** The completely white hair that re–grew on a shaved patch of a *bcl–2*$^{-/-}$ *bim*$^{+/-}$ mouse (re–grown hair remained entirely black on *bcl–2*$^{+/-}$ *bim*$^{+/-}$ mice and nearly all black on the *bcl–2*$^{-/-}$ *bim*$^{-/-}$ animals). **d,** Expression of β–galactosidase in a heterozygous *bim–β–gal* 8-wk old mouse in the outer root sheath (ors) of the hair follicle (x 15). Sections in **a,b** were stained with hematoxylin and eosin; that in **d** was stained for X–gal and counterstained with eosin.

Figure 4 is a graphical representation showing that absence of Bim restores a robust haemopoietic system in Bcl–2–deficient mice. **a,** Numbers of total leukocytes, B

cells (B220$^+$), T cells (Thy.1$^+$) and myeloid cells (macrophages plus granulocytes; Mac–1$^+$ + Gr–1$^+$), expressed as mean ± SD, in the spleen of 8 – 10 wk–old mice (N= 5 – 10 mice per genotype). The data on *bcl–2*$^{-/-}$ cells represent numbers of cells of donor origin in Ly5.1 congenic wt mice irradiated and reconstituted 2 months earlier with *bcl–2*$^{-/-}$ Ly5.2 foetal liver cells. The effects of genotype were similar in blood and lymph nodes. **b,** Flow cytometric analyses of lymph node cells from reconstituted mice, showing the poor maintenance of *bcl–2*$^{-/-}$ lymphocytes compared to *bcl–2*$^{-/-}$ *bim*$^{-/-}$ and wt cells. Six months after reconstitution, the lymph node cells were stained with antibodies to Ly5.2, to distinguish donor–derived cells from those of host (Ly5.1) origin, and to the B220 marker, to separate B cells (B220$^+$) from T cells (B220$^-$). Total cell numbers in the lymph nodes (Ly5.1 plus Ly5.2) are indicated, as well as the percentages of different cell types (N> 3 mice per genotype).

Figure 5 is a graphical representation showing opposing actions of Bcl–2 and Bim on lymphocyte survival in culture. **a,** Mature B cells (IgM^{low}lgD^{high}) purified from the lymph nodes were cultured without cytokine support for six days and viability determined daily (Mean ± SD). **b,** CD8$^+$ T cells, treated similarly. CD4$^+$ T cells gave equivalent results. **c,** Mature B and T cells of the indicated genotypes were treated with etoposide (VP16, at 10 or 1 μg/mL) for 24 or 72 h. Dexamethasone (1 μM for 24 h) gave similar effects. Since the lymphopenic *bcl–2*$^{-/-}$ mice provided insufficient cells for these experiments, the equivalent (donor–derived) cells were purified from the reconstituted mice. All experiments were done in duplicate on at least 3 mice of each genotype.

Figure 6 is a graphical representation showing that survival signal from IL–7 requires Bcl–2 even in the absence of Bim. Mature CD8$^+$ T cells from lymph nodes of mice of the indicated genotypes were cultured in the presence of an optimal concentration of IL–7 for 6 days and the viability of cells determined daily (Mean + SD), All experiments were done in duplicate on at least 3 mice of each genotype.

BRIEF DESCRIPTION OF THE SEQUENCES: SUMMARY TABLE

TABLE A

SEQUENCE ID NUMBER	SEQUENCE	LENGTH
SEQ ID NO: 1	Nucleotide sequence corresponding to the mouse *bcl–2* gene as set forth in GenBank Accession No. M16506	2914 nts
SEQ ID NO: 2	Bcl–2 α polypeptide encoded by SEQ ID NO: 1	236 aa
SEQ ID NO: 3	Bcl–2 β polypeptide encoded by SEQ ID NO: 1	199 aa
SEQ ID NO: 4	Nucleotide sequence corresponding to the mouse *bim* promoter sequence	795 nts
SEQ ID NO: 5	Nucleotide sequence corresponding to the mouse *bimL* cDNA	423 nts
SEQ ID NO: 6	BimL polypeptide encoded by SEQ ID NO: 5	140 aa
SEQ ID NO: 7	Nucleotide sequence corresponding to the mouse *bimEL* cDNA	591 nts
SEQ ID NO: 8	BimEL polypeptide encoded by SEQ ID NO: 7	196 aa
SEQ ID NO: 9	Sense primer for detection of wt *bcl–2* allele	25 nts
SEQ ID NO: 10	Antisense primer for detection of wt *bcl–2* allele	25 nts
SEQ ID NO: 11	Sense primer for detection of mutant *bcl–2* allele	25 nts
SEQ ID NO: 12	Antisense primer for detection of mutant *bcl–2* allele	21 nts
SEQ ID NO: 13	Sense primer for detection of wt *bim* allele	21 nts
SEQ ID NO: 14	Antisense primer for detection of wt *bim* allele	21 nts
SEQ ID NO: 15	Sense primer for detection of null *bim* allele	21 nts
SEQ ID NO: 16	Antisense primer for detection of null *bim* allele	20 nts
SEQ ID NO: 17	Sense primer for detection of wt *bim* allele	21 nts
SEQ ID NO: 18	Antisense primer for detection of wt *bim* allele	21 nts
SEQ ID NO: 19	β–gal specific sense primer	21 nts
SEQ ID NO: 20	Anti-sense primer located in *bim* intron 2	22 nts

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

The articles "*a*" and "*an*" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

By "*agent*" is meant a naturally occurring or synthetically produced molecule which interacts either directly or indirectly with a target member, the level and/or functional activity of which is to be modulated.

Throughout this specification, unless the context requires otherwise, the words "*comprise*", "*comprises*" and "*comprising*" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

By "*corresponds to*" or "*corresponding to*" is meant (a) a polynucleotide having a nucleotide sequence that is substantially identical or complementary to all or a portion of a reference polynucleotide sequence or encoding an amino acid sequence identical to an amino acid sequence in a peptide or protein; or (b) a peptide or polypeptide having an amino acid sequence that is substantially identical to a sequence of amino acids in a reference peptide or protein.

The term "*complementary*" refers to the topological capability or matching together of interacting surfaces of a pair of polynucleotides. Complementary includes base complementarity such as A is complementary to T or U, and C is complementary to G in the genetic code. However, this invention also encompasses situations in which there is non-traditional base-pairing such as Hoogsteen base pairing which has been identified in certain transfer RNA molecules and postulated to exist in a triple helix. In the context of the definition of the term "complementary", the terms "match" and "mismatch" as used

herein refer to the hybridisation potential of paired nucleotides in complementary nucleic acid strands. Matched nucleotides hybridise efficiently, such as the classical A-T and G-C base pair mentioned above. Mismatches are other combinations of nucleotides that hybridise less efficiently. The term "complementary", therefore, includes within its scope 'exact complementarity" and 'substantially complementary', in which a reference polynucleotide is sufficiently complementary to hybridise with a target nucleotide sequence.

By "*effective amount*", in the context of modulating an activity or of treating or preventing a condition associated with the activation or inactivation of apoptosis is meant the administration of that amount of active to an individual in need of such modulation, treatment or prophylaxis, either in a single dose or as part of a series, that is effective for modulating that effect or for treating or preventing that condition. The effective amount will vary depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The terms "*foreign polynucleotide*" or "*exogenous polynucleotide*" or "*heterologous polynucleotide*" and the like refer to any nucleic acid (e.g., gene sequence) which is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene contains some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, the presence of a loxP site, etc.) relative to the naturally-occurring gene.

The term "*gene*" as used herein refers to any and all discrete coding regions of the cell's genome, as well as associated non-coding and regulatory regions. Thus, the terms "*bcl–2* gene" and "*bim* gene" are used generically herein to designate respectively *bcl–2* genes and *bim* genes, *e.g.* variants from rat, human, mouse, guinea pig, etc., and their alternate forms. The term "*gene*" is also intended to mean the open reading frame encoding specific polypeptides, introns, and adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 1 kb beyond the coding region, but possibly further in either direction. In this regard, the gene may further comprise endogenous (*i.e.*, naturally associated with a given gene) or heterologous control signals such as promoters, enhancers, termination and/or polyadenylation signals. The DNA sequences encoding *bcl–2* or *bim* may be cDNA or genomic DNA or a fragment thereof.

The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

The term "*homologous*" as used herein denotes a characteristic of a DNA sequence having at least about 70 percent sequence identity as compared to a reference sequence, typically at least about 85 percent sequence identity, preferably at least about 95 percent sequence identity, and more preferably about 98 percent sequence identity, and most preferably about 100 percent sequence identity as compared to a reference sequence. Homology can be determined using, for example, a "BLASTN" algorithm. It is understood that homologous sequences can accommodate insertions, deletions and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome.

By "*isolated*" is meant material that is substantially or essentially free from components that normally accompany it in its native state.

As used herein the term "*level*" refers to a concentration or amount of a molecule or substance or group of molecules or substances in a cell or sample.

By "*modulating*" is meant increasing or decreasing, either directly or indirectly, the level and/or functional activity of a target molecule. For example, an agent may indirectly modulate the level/activity by interacting with a molecule other than the target molecule. In this regard, indirect modulation of a gene encoding a target polypeptide includes within its scope modulation of the expression of a first nucleic acid molecule, wherein an expression product of the first nucleic acid molecule modulates the expression of a nucleic acid molecule encoding the target polypeptide.

The term "*oligonucleotide*" as used herein refers to a polymer composed of a multiplicity of nucleotide units (deoxyribonucleotides or ribonucleotides, or related structural variants or synthetic analogues thereof) linked via phosphodiester bonds (or related structural variants or synthetic analogues thereof). Thus, while the term "oligonucleotide" typically refers to a nucleotide polymer in which the nucleotides and linkages between them are naturally occurring, it will be understood that the term also includes within its scope various analogues including, but not restricted to, peptide nucleic acids (PNAs), phosphoramidates, phosphorothioates, methyl phosphonates, 2-O-methyl

ribonucleic acids, and the like. The exact size of the molecule may vary depending on the particular application. An oligonucleotide is typically rather short in length, generally from about 10 to 30 nucleotides, but the term can refer to molecules of any length, although the term "polynucleotide" or "nucleic acid" is typically used for large oligonucleotides.

The term "*patient*" refers to patients of human or other animal and includes any individual it is desired to examine or treat using the methods of the invention. However, it will be understood that "*patient*" does not imply that symptoms are present. Suitable animals that fall within the scope of the invention include, but are not restricted to, primates, livestock animals (*e.g.*, sheep, cows, horses, donkeys, pigs), laboratory test animals (*e.g.*, rabbits, mice, rats, guinea pigs, hamsters), companion animals (*e.g.*, cats, dogs) and captive wild animals (*e.g.*, kangaroo, koala, foxes, deer, dingoes), aves (*e.g.*, chicken, geese, duck, emu, ostrich), reptile or fish.

By "*pharmaceutically-acceptable carrier*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in topical or systemic administration.

The term "*polynucleotide*" or "*nucleic acid*" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to oligonucleotides greater than 30 nucleotides in length. Polynucleotide sequences are understood to encompass complementary strands as well as alternative backbones described herein.

"*Polypeptide*", "*peptide*" and "*protein*" are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers.

By "*primer*" is meant an oligonucleotide which, when paired with a strand of DNA, is capable of initiating the synthesis of a primer extension product in the presence of a suitable polymerising agent. The primer is preferably single-stranded for maximum efficiency in amplification but may alternatively be double-stranded. A primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerisation agent. The length of the primer depends on many factors, including application, temperature to be employed, template reaction conditions, other reagents, and source of primers. For example, depending on the complexity of the target sequence, the

oligonucleotide primer typically contains 15 to 35 or more nucleotides, although it may contain fewer nucleotides. Primers can be large polynucleotides, such as from about 200 nucleotides to several kilobases or more. Primers may be selected to be "substantially complementary" to the sequence on the template to which it is designed to hybridise and serve as a site for the initiation of synthesis. By "substantially complementary", it is meant that the primer is sufficiently complementary to hybridise with a target nucleotide sequence. Preferably, the primer contains no mismatches with the template to which it is designed to hybridise but this is not essential. For example, non-complementary nucleotides may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary nucleotides or a stretch of non-complementary nucleotides can be interspersed into a primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridise therewith and thereby form a template for synthesis of the extension product of the primer.

By "*substantially complete loss of function*" is meant at least 90%, usually at least 95% and more usually at least 99% loss of function.

The term "*transgene*" is used herein to describe genetic material that has been or is about to be artificially inserted into the genome of a cell, particularly a mammalian cell of a living animal. The transgene is used to transform a cell, meaning that a permanent or transient genetic change, preferably a permanent genetic change, is induced in a cell following incorporation of exogenous DNA. A permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. Of interest are non-human animals including vertebrates, preferably mammals such as rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.

As used herein, the terms "*selectable marker gene*" and "*positive selection marker gene*" refer to a gene encoding a product that enables only the cells that carry the gene to survive and/or grow under certain conditions. For example, plant and animal cells that express the introduced neomycin resistance (Neo^r) gene are resistant to the compound G418. Cells that do not carry the Neo^r gene marker are killed by G418. Other positive selection markers are known to or are within the purview of those of ordinary skill in the art.

By "*vector*" is meant a nucleic acid molecule, preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, or plant virus, into which a nucleic acid sequence may be inserted or cloned. A vector preferably contains one or more unique restriction sites and may be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a linear or closed circular plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. A vector system may comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants. Examples of such resistance genes are well known to those of skill in the art.

The term "*wild-type*" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "*normal*" or "*wild-type*" form of the gene. In contrast, the term "*modified*" "*variant*" or "*mutant*" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

As used herein, underscoring or italicising the name of a gene shall indicate the gene, in contrast to its protein product, which is indicated by the name of the gene in the absence of any underscoring or italicising. For example, "*bcl–2*" shall mean the *bcl–2* gene, whereas "Bcl–2" shall indicate the protein product of the "*bcl–2*" gene.

2. Modulation of BH3–only proteins

The present invention is predicated in part on the determination that Bim levels set the threshold for initiation of apoptosis in several tissues and more generally that apoptosis and homeostasis are tightly regulated by the balance between BH3–only proteins and their pro-survival relatives. Thus, in accordance with the present invention, it is proposed that disease progression in degenerative disorders characterised by inappropriate cell death can be halted by drugs that inhibit particular BH3–only proteins or their upstream regulators or that mimic the action of their antagonists (*e.g.*, mimic the action of Bcl-2 when the BH3-only protein is Bim). It is further proposed that disease progression in degenerative disorders characterised by inappropriate cell proliferation such as cancer and autoimmune disease can be ameliorated by drugs that either up–regulate the activity of BH3–only proteins or their upstream regulators or mimic their action on their pro–survival counterparts.

Accordingly, one aspect of the present invention contemplates the use of modulators of BH3–only proteins for the treatment and/or prevention of degenerative disorders, including disorders characterised by inappropriate cell proliferation or inappropriate cell death or in some cases, both. The BH3–only protein is preferably selected from BAD, BID, Hrk/DP5, Bik/Nbk, Blk, Bmf, NOXA, PUMA or Bim, or variants thereof. In a preferred embodiment, the BH3–only protein is Bim.

Premature widespread apoptosis (inappropriate cell death) causes much of the damage associated with degenerative disorders including, for example, ischaemic disease (*e.g.*, myocardial infarction), neurodegenerative disease, peripheral nerve injury, aplastic anaemia, liver damage, chemotherapy, irradiation (*e.g.*, γ-irradiation), tissue atrophy and HIV or other viral infections. In accordance with the present invention, degenerative disorders of this type may be treated or prevented by the administration of BH3–only protein antagonists or antagonists of their upstream regulators, which suppress apoptosis.

Examples of ischaemic diseases include heart disease such as cardiac infarction, cardiac angina, congestive heart failure and arrhythmia, and cerebrovascular diseases such as cerebral stroke, subarachnoidal haemorrhage, cerebral infarction, and cerebral thrombosis.

Examples of the neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, pigmented retinitis, and cerebelli

degeneration. In this regard, antagonists of BH3–only proteins or of their upstream regulators can be used to treat or prevent degenerative disorders due to premature or excessive cell loss during aging which can lead to organ dysfunction and disease. Such degenerative disorders include degenerative diseases of the central nervous system due to aging or other factors which result in the death of neurones.

Degenerative disorders characterised by inappropriate cell proliferation include cancer, autoimmune disorders, tissue hypertrophy, and inflammatory disorders including inflammation arising from acute tissue injury including, for example, acute lung injury. In accordance with the present invention, such degenerative disorders may be treated or prevented by the administration of BH3–only protein agonists or mimics.

Many types of cancer can be treated by the administration of agonists or mimics of BH3–only proteins, including for example, carcinomas, sarcomas, and leukemia/lymphomas, including for example, carcinomas such as adenocarcinomas, squamous carcinomas, carcinoma of the organs including breast, colon, head, neck, etc.; sarcomas including chondrosarcoma, melanosarcoma, to etc.; and leukemia and lymphomas including acute lymphomatic leukemia, acute myelogenous leukemia, non-Hodgkin's lymphoma, Burkitt's lymphoma, B-cell lymphomas, T-cell lymphomas, etc.

Agonists or mimics of BH3–only proteins can also be used for treatment of autoimmune disorders by inducing apoptosis in autoreactive T lymphocytes, for example, in patients suffering systemic lupus erythematosus. Other autoimmune diseases amenable to treatment by suppressing or inducing apoptosis through the administration of such agents include, for example, rheumatoid arthritis, myasthenia gravis, Grave's disease, Hashimoto's thyroiditis, insulin-resistant diabetes, allergic rhinitis, asthma, functional autonomic abnormalities, juvenile insulin-dependent diabetes, Addison's disease, idiopathic hypoparathyroidism, spontaneous infertility, premature ovarian failure, pemphigus, Bullous pemphigoid, primary biliary cirrhosis, autoimmune haemolytic anaemia, idiopathic thrombocytopenic purpura, idiopathic neutropenia, Goodpasture's syndrome, rheumatoid arthritis and Sjogren's syndrome.

Agonists or mimics of BH3–only proteins can be used to treat inflammation resulting from acute lung injury, by inducing apoptosis. The disease process begins with an explosive inflammatory response in the alveolar wall. In the aftermath of the resulting tissue destruction, extensive fibroproliferation of the alveolar air space ensues, consisting

Fukuda *et al.* (1987, *Am. J. Pathol.* **126**: 171-182). An important mechanism for the systematic elimination of the foregoing is apoptosis, *i.e.*, programmed cell death.

Agents that may be used to decrease the level and/or functional activity of a BH3–only protein include any suitable gene or expression product inhibitor which can be identified or produced by conventional protocols known in the art as for example disclosed in Section 3 *infra* or using the transgenic non–human animal model of the invention. Suitable agents which can be used to reduce or abrogate expression of a gene encoding a BH3–only protein include, but are not restricted to, oligoribonucleotide sequences, including antisense RNA and DNA molecules and ribozymes, that function to inhibit the translation, for example, of BH3–only protein–encoding mRNA. Antisense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between -10 and +10 regions of an BH3–only protein gene, are preferred. When employed, antisense RNAs should be at least about 10-20 nucleotides or greater in length, and be at least about 75% complementary to their target genes or gene transcripts such that expression of the targeted homologous sequence is precluded.

Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridisation of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyse endonucleolytic cleavage of calpain RNA sequences. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. When employed, ribozymes may be selected from the group consisting of hammerhead ribozymes, axehead ribozymes, newt satellite ribozymes, Tetrahymena ribozymes and RNAse P, and are designed according to methods known in the art based on the sequence of the target gene (for instance, see U.S. Pat. No. 5,741,679). The suitability of candidate

targets may also be evaluated by testing their accessibility to hybridisation with complementary oligonucleotides, using ribonuclease protection assays.

Both antisense RNA and DNA molecules and ribozymes may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesising oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesise antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines. Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

Preferred agents that may be used to decrease the level and/or functional activity of a BH3–only protein include RNA molecules that mediate RNA interference (RNAi) of a target gene or gene transcript. RNAi refers to interference with or destruction of the product of a target gene by introducing a single stranded, and typically a double stranded RNA (dsRNA), that is homologous to the transcript of a target gene. Thus, in one embodiment, dsRNA *per se* and especially dsRNA-producing constructs corresponding to at least a portion of a BH3-only protein may be used to decrease its level and/or functional activity. RNAi-mediated inhibition of gene expression may be accomplished using any of the techniques reported in the art, for instance by transfecting a nucleic acid construct encoding a stem-loop or hairpin RNA structure into the genome of the target cell, or by expressing a transfected nucleic acid construct having homology for a target gene from between convergent promoters, or as a head to head or tail to tail duplication from behind a single promoter. Any similar construct may be used so long as it produces a single RNA having the ability to fold back on itself and produce a dsRNA, or so long as it produces two separate RNA transcripts which then anneal to form a dsRNA having homology to a target gene.

Absolute homology is not required for RNAi, with a lower threshold being described at about 85% homology for a dsRNA of about 200 base pairs (Plasterk and Ketting, 2000, *Current Opinion in Genetics and Dev.* **10**: 562-67). Therefore, depending on the length of the dsRNA, the RNAi-encoding nucleic acids can vary in the level of homology they contain toward the target gene transcript, *i.e.*, with dsRNAs of 100 to 200 base pairs having at least about 85% homology with the target gene, and longer dsRNAs, *i.e.*, 300 to 100 base pairs, having at least about 75% homology to the target gene. RNA-encoding constructs that express a single RNA transcript designed to anneal to a separately expressed RNA, or single constructs expressing separate transcripts from convergent promoters, are preferably at least about 100 nucleotides in length. RNA-encoding constructs that express a single RNA designed to form a dsRNA *via* internal folding are preferably at least about 200 nucleotides in length.

The promoter used to express the dsRNA-forming construct may be any type of promoter if the resulting dsRNA is specific for a gene product in the cell lineage targeted for destruction. Alternatively, the promoter may be lineage specific in that it is only expressed in cells of a particular development lineage. This might be advantageous where some overlap in homology is observed with a gene that is expressed in a non-targeted cell lineage. The promoter may also be inducible by externally controlled factors, or by intracellular environmental factors.

In another embodiment, RNA molecules of about 21 to about 23 nucleotides, which direct cleavage of specific mRNA to which they correspond, as for example described by Tuschl *et al.* in U.S. Patent Application No. 20020086356, can be utilised for mediating RNAi. Such 21-23 nt RNA molecules can comprise a 3' hydroxyl group, can be single-stranded or double stranded (as two 21-23 nt RNAs) wherein the dsRNA molecules can be blunt ended or comprise overhanging ends (e.g., 5', 3').

Agents that may be used to enhance the activity of a BH3–only protein include any suitable BH3–only protein inducer or stabilising/activating agent which can be identified or produced by standard protocols as for example disclosed in Section 3 *infra* or using the transgenic non–human animal model of the invention. In this instance, the agent may comprise a BH3–only protein or polynucleotide encoding same. .

3. *Identification of BH3–only protein modulators*

The invention also features a method of screening for an agent that modulates the expression of a gene or the level and/or functional activity of an expression product of the gene, wherein the gene is selected from a gene encoding a BH3–only protein, or a gene belonging to the same regulatory or biosynthetic pathway as a BH3–only protein–encoding gene, or a variant of the gene. The method comprises contacting a preparation comprising a first member selected from the expression product, or a biologically active fragment of the expression product, or a second member selected from a genetic sequence that regulates or encodes the expression product or a fragment of the genetic sequence, with a test agent, and detecting a change in the level and/or functional activity of the first member, or of an expression product derived from, or relating to, the second member.

Any suitable assay for detecting, measuring or otherwise determining the the modulation or modulation of apoptosis is contemplated by the present invention. Assays of a suitable nature are known to persons of skill in the art.

Modulators contemplated by the present invention include agonists and antagonists of gene expression including antisense molecules, ribozymes and co-suppression molecules, as for example described in Section 2. Agonists include molecules which increase promoter activity or interfere with negative mechanisms. Agonists of a BH3–only protein–encoding gene include molecules which overcome any negative regulatory mechanism. Antagonists of BH3–only proteins include antibodies and inhibitor peptide fragments.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 Dalton. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogues or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomised oligonucleotides and oligopeptides. Such agents include, but are not limited to, peptides such as, for example, soluble peptides, including, but not restricted to, members of random peptide libraries (see, *e.g.*, Lam *et al.*, 1991, *Nature* **354**: 82-84; Houghten *et al.*, 1991, *Nature* **354**: 84-86), and combinatorial chemistry-derived molecular library peptides made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited, to members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang *et al.*, 1993, *Cell* **72**: 767-778); antibodies (including, but not limited to, polyclonal, monoclonal, humanised, anti-idiotypic, chimeric or single chain antibodies, and Fab, $F(ab)_2$ and Fab expression library fragments, and epitope-binding fragments thereof).

Other compounds which can be screened in accordance with the invention include but are not limited to small organic molecules that are able to gain entry into an appropriate cell and affect the expression of a gene (*e.g.*, by interacting with the regulatory region or transcription factors involved in gene expression); or such compounds that affect the activity of a gene by inhibiting or enhancing the binding of accessory molecules.

Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogues.

Screening may also be directed to known pharmacologically active compounds and chemical analogues thereof.

Any suitable screening method is contemplated by the present invention. For example, the method may include contacting a cell comprising a polynucleotide corresponding to an BH3–only protein–encoding gene or a gene belonging to the same regulatory or biosynthetic pathway as a BH3–only protein–encoding gene, with an agent suspected of having the modulatory activity and screening for the modulation of the level

the level of an expression product encoded by the polynucleotide, or the modulation of the activity or expression of a downstream cellular target of the protein or of the expression product. Detecting such modulation can be achieved utilising techniques including, but not restricted to, ELISA, cell-based ELISA, filter-binding ELISA, inhibition ELISA, flow cytometry including fluorescence activated cell sorting (FACS), Western blots, immunoprecipitation, slot or dot blot assays, immunostaining, RIA, scintillation proximity assays, fluorescent immunoassays using antigen-binding molecule conjugates or antigen conjugates of fluorescent substances such as fluorescein or rhodamine, Ouchterlony double diffusion analysis, immunoassays employing an avidin-biotin or a streptavidin-biotin detection system, and nucleic acid detection assays including reverse transcriptase polymerase chain reaction (RT-PCR).

It will be understood that a polynucleotide from which an expression product of interest is regulated or expressed may be naturally occurring in the cell which is the subject of testing or it may have been introduced into the host cell for the purpose of testing. Further, the naturally-occurring or introduced polynucleotide may be constitutively expressed – thereby providing a model useful in screening for agents which downregulate expression of an encoded product of the sequence wherein the down regulation can be at the nucleic acid or expression product level – or may require activation – thereby providing a model useful in screening for agents that upregulate expression of an encoded product of the sequence. Further, to the extent that a polynucleotide is introduced into a cell, that polynucleotide may comprise the entire coding sequence which codes for a target protein or it may comprise a portion of that coding sequence (*e.g.*, at least a portion of a BH3 domain) or a portion that regulates expression of a product encoded by the polynucleotide (*e.g.*, a promoter). For example, the promoter that is naturally associated with the polynucleotide may be introduced into the cell that is the subject of testing. In this regard, where only the promoter is utilised, detecting modulation of the promoter activity can be achieved, for example, by operably linking the promoter to a suitable reporter polynucleotide including, but not restricted to, green fluorescent protein (GFP), luciferase, β-galactosidase and catecholamine acetyl transferase (CAT). Modulation of expression may be determined by measuring the activity associated with the reporter polynucleotide.

In another example, the subject of detection could be a downstream regulatory target of the molecule to be targeted (target molecule), rather than target molecule itself or

the reporter molecule operably linked to a promoter of a gene encoding a product the expression of which is regulated by the target molecule.

These methods provide a mechanism for performing high throughput screening of putative modulatory agents such as proteinaceous or non-proteinaceous agents comprising synthetic, combinatorial, chemical and natural libraries. These methods will also facilitate the detection of agents which bind either the polynucleotide encoding the target molecule or which modulate the expression of an upstream molecule, which subsequently modulates the expression of the polynucleotide encoding the target molecule. Accordingly, these methods provide a mechanism of detecting agents that either directly or indirectly modulate the expression and/or activity of a target molecule according to the invention.

In a series of preferred embodiments, the present invention provides assays for identifying small molecules or other compounds (*i.e.*, modulatory agents) which are capable of inducing or inhibiting the level and/or or functional activity of target molecules according to the invention. The assays may be performed *in vitro* using non-transformed cells, immortalised cell lines, or recombinant cell lines. In addition, the assays may detect the presence of increased or decreased expression of genes or production of proteins on the basis of increased or decreased mRNA expression (using, for example, the nucleic acid probes disclosed herein), increased or decreased levels of protein products (using, for example, the antigen binding molecules disclosed herein), or increased or decreased levels of expression of a reporter gene (*e.g.*, GFP, β-galactosidase or luciferase) operably linked to a target molecule-related gene regulatory region in a recombinant construct.

Thus, for example, one may culture cells which produce a particular target molecule and add to the culture medium one or more test compounds. After allowing a sufficient period of time (*e.g.*, 6-72 hours) for the compound to induce or inhibit the level and/or functional activity of the target molecule, any change in the level from an established baseline may be detected using any of the techniques described above and well known in the art. In particularly preferred embodiments, the cells are selected from kidney cells, lymphocytes, melanocytes or neural cells. Using appropriate nucleic acid probes and/or antigen-binding molecules , detection of changes in the level and or functional activity of a target molecule, and thus identification of the compound as agonist or antagonist of the target molecule, requires only routine experimentation.

In particularly preferred embodiments, a recombinant assay is employed in which a reporter gene encoding, for example, GFP, β-galactosidase or luciferase is operably linked to the 5' regulatory regions of a target molecule related gene. Such regulatory regions may be easily isolated and cloned by one of ordinary skill in the art. The reporter gene and regulatory regions are joined in-frame (or in each of the three possible reading frames) so that transcription and translation of the reporter gene may proceed under the control of the regulatory elements of the target molecule related gene. The recombinant construct may then be introduced into any appropriate cell type although mammalian cells are preferred, and human cells are most preferred. The transformed cells may be grown in culture and, after establishing the baseline level of expression of the reporter gene, test compounds may be added to the medium. The ease of detection of the expression of the reporter gene provides for a rapid, high throughput assay for the identification of agonists or antagonists of the target molecules of the invention.

Compounds identified by this method will have potential utility in modifying the expression of target molecule related genes *in vivo*. These compounds may be further tested in the animal models to identify those compounds having the most potent *in vivo* effects. In addition, as described above with respect to small molecules having target polypeptide binding activity, these molecules may serve as "lead compounds" for the further development of pharmaceuticals by, for example, subjecting the compounds to sequential modifications, molecular modelling, and other routine procedures employed in rational drug design.

In another embodiment, random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to a target molecule or to a functional domain thereof. Identification of molecules that are able to bind to a target molecule may be accomplished by screening a peptide library with a recombinant soluble target molecule. The target molecule may be purified, recombinantly expressed or synthesised by any suitable technique. Such molecules may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook, *et al.*, (1989, *supra*) in particular Sections 16 and 17; Ausubel *et al.*, (1994-1998, *supra*), in particular Chapters 10 and 16; and Coligan *et al.*, (1995-1997, *supra*), in particular Chapters 1, 5 and 6. Alternatively, a target polypeptide according to the invention may be synthesised using

solution synthesis or solid phase synthesis as described, for example, in Chapter 9 of Atherton and Shephard (*supra*) and in Roberge *et al* (1995, *Science* **269**: 202).

To identify and isolate the peptide/solid phase support that interacts and forms a complex with a target molecule, preferably a target polypeptide, it may be necessary to label or "tag" the target polypeptide. The target polypeptide may be conjugated to any suitable reporter molecule, including enzymes such as alkaline phosphatase and horseradish peroxidase and fluorescent reporter molecules such as fluorescein isothyiocynate (FITC), phycoerythrin (PE) and rhodamine. Conjugation of any given reporter molecule, with target polypeptide, may be performed using techniques that are routine in the art. Alternatively, target polypeptide expression vectors may be engineered to express a chimeric target polypeptide containing an epitope for which a commercially available antigen-binding molecule exists. The epitope specific antigen-binding molecule may be tagged using methods well known in the art including labelling with enzymes, fluorescent dyes or coloured or magnetic beads.

For example, the "tagged" target polypeptide conjugate is incubated with the random peptide library for 30 minutes to one hour at 22° C to allow complex formation between target polypeptide and peptide species within the library. The library is then washed to remove any unbound target polypeptide. If the target polypeptide has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing a substrate for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diamnobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-target polypeptide complex changes colour, and can be easily identified and isolated physically under a dissecting microscope with a micromanipulator. If a fluorescently tagged target polypeptide has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric target polypeptide having a heterologous epitope has been used, detection of the peptide/target polypeptide complex may be accomplished by using a labelled epitope specific antigen-binding molecule. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

4. *Genetically modified animals of the invention*

The invention also provides genetically modified, non-human animals having a partial or complete loss of function in one or both alleles of the endogenous *bcl–2* gene and

a partial or complete loss of function in one or both alleles of the endogenous *bim* gene. These partial or complete loss of functions are suitably the result of an alteration to the *bcl–2* gene and/or to a *bim* gene, which include, but are not restricted to, deletions or other loss of function mutations, introduction of an exogenous gene having a nucleotide sequence with targeted or random mutations, introduction of an exogenous gene from another species, or a combination thereof. The genetically modified animal may be either homozygous or heterozygous for the alteration.

The genetically modified animals of the present invention typically, but not exclusively, comprise a foreign or exogenous polynucleotide sequence or transgene present as an extrachromosomal element or stably integrated in all or a portion of its cells, especially in germ cells. Unless otherwise indicated, it will be assumed that a genetically modified animal comprises stable changes to the germline sequence. During the initial construction of the animal, "chimeras" or "chimeric animals" are generated, in which only a subset of cells have the altered genome. Chimeras are primarily used for breeding purposes in order to generate the desired genetically modified animal. Animals having a heterozygous alteration are generated by breeding of chimeras. Male and female heterozygotes are typically bred to generate homozygous animals.

Since the genetically modified animals of the invention have a partial or complete loss of function in one or both alleles of the endogenous *bcl–2* gene and of the endogenous *bim* gene, they fall into the group of genetically modified animals colloquially termed "knockouts". A knockout may be achieved by a variety of mechanisms, including introduction of a disruption of the coding sequence, *e.g.* insertion of one or more stop codons, insertion of a DNA fragment, etc., deletion of coding sequence, substitution of stop codons for coding sequence, etc. In some cases the foreign transgene sequences are ultimately deleted from the genome, leaving a net change to the native sequence. Different approaches may be used to achieve the "knockout". A chromosomal deletion of all or part of the native *bcl–2* or *bim* may be induced, including deletions of the non-coding regions, particularly the promoter region, 3' regulatory sequences, enhancers, or deletion of a gene that activates expression of *bcl–2* or *bim*. A functional knockout may also be achieved by the introduction of an anti-sense construct that blocks expression of the native *bcl–2* and/or *bim* genes (for example, see Li and Cohen, 1996, *Cell* **85**: 319-329). "Knockouts" also include conditional knock-outs, for example where alteration of the target gene occurs upon exposure of the animal to a substance that promotes target gene alteration,

introduction of an enzyme that promotes recombination at the target gene site (e.g. Cre in the Cre-lox system), or other method for directing the target gene alteration postnatally.

In a preferred embodiment, the partial or complete loss of function in one or both alleles of *bcl–2* or *bim* gene is effected by disruption of that gene. Accordingly, the genetically modified animal preferably comprises a disruption in at least one allele of the endogenous *bim* gene and a disruption in at least one allele of the endogenous *bcl–2* gene. In accordance with the present invention, the disruption suitably results in an inability of the animal to produce a corresponding functional expression product or detectable levels of the expression product. Accordingly, a disruption in the endogenous *bcl–2* gene may result in a reduced level and/or functional activity of Bcl–2 or in an inability of the animal to produce a functional Bcl–2 or detectable levels of Bcl–2 relative to a corresponding animal without the disruption. Similarly, a disruption in the endogenous *bim* gene may result in a reduced level and/or functional activity of Bim or in an inability of the animal to produce a functional Bim or detectable levels of Bim relative to a corresponding animal without the disruption.

A disruption typically comprises an insertion of a nucleic acid sequence into one region of the native genomic sequence (usually one or more exons) and/or the promoter region of a gene so as to decrease or prevent expression of that gene in the cell as compared to the wild-type or naturally occurring sequence of the gene. By way of example, a nucleic acid construct can be prepared containing a selectable marker gene which is inserted into a targeting nucleic acid sequence that is complementary to a genomic sequence (promoter and/or coding region) to be disrupted. Useful genomic sequences to be disrupted include, but are not restricted to, *bcl–2* or *bim* open reading frames encoding polypeptides or domains, introns, and adjacent 5' and 3' non-coding nucleotide sequences involved in regulation of gene expression. Accordingly, a targeting sequence may comprise some or part of the nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including some or all of the introns that are normally present in a native chromosome. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, etc., including about 1 kb, but possibly more, of flanking genomic DNA at either the 5' or 3' end of the transcribed region. When the nucleic acid construct is then transfected into a cell, the construct will integrate into the genomic DNA. Thus, many progeny of the cell will no

longer express the gene at least in some cells, or will express it at a decreased level, as the genomic sequence is now disrupted by the selection marker.

The disruption in the endogenous *bim* gene suitably comprises a deletion of a *bim* nucleotide sequence encoding at least a portion of a Bim polypeptide. For example, the disruption may comprise a deletion of the first three coding exons (*i.e.*, exons 2, 3 and 4, Bouillet *et al*, 2001) of the endogenous *bim* gene. Preferably, the disruption comprises a deletion of the BH3-encoding exon (*i.e.*, exons 5, Bouillet *et al*, 2001) of the endogenous *bim* gene.

The disruption in the endogenous *bcl–2* gene suitably comprises a deletion of a *bcl–2* nucleotide sequence encoding at least a portion of a Bcl–2 polypeptide. Preferably, the disruption comprises a deletion of the entire coding regions for the *bcl–2*α and *bcl–2*β transcripts (Nakayama *et al.*, 1994; Nakayama *et al.*, 1993) of the endogenous *bcl–2* gene.

In another embodiment, an individual disruption reduces, abrogates or otherwise impairs the expression of a corresponding gene and in this regard, the disruption may reside in the deletion of at least a portion of the transcriptional and/or translational regulatory sequences associated with that gene.

Specific examples of the genetically modified animals of the present invention include those containing:

(a) a substantially complete loss of function in a single allele of the endogenous *bim* gene and a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene (*i.e.*, $bim^{+/-}$ $bcl\text{-}2^{-/-}$);

(b) a substantially complete loss of function in a single allele of the endogenous *bim* gene and a substantially complete loss of function in a single allele of the endogenous *bcl–2* gene (*i.e.*, $bim^{+/-}$ $bcl\text{-}2^{+/-}$);

(c) a substantially complete loss of function in both alleles of the endogenous *bim* gene and a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene (*i.e.*, $bim^{-/-}$ $bcl\text{-}2^{-/-}$); or

(d) genetic or functional equivalents of (a), (b) or (c).

Suitable genetic or functional equivalent animals include those containing anti-sense constructs comprising a sequence complementary to at least a portion of an endogenous *bcl–2* gene or to at least a portion of an endogenous *bim* gene which will block expression of a corresponding expression product to a level analogous to that in (a), (b) or

(c) above. Alternatively, a genetic or functional equivalent animal may contain a partial or complete loss of function in both alleles of an endogenous *bcl–2* gene and/or in both alleles of an endogenous *bim* gene, together with an exogenous sequence (*e.g.*, *bcl–2* cDNA and/or *bim* cDNA), from which Bcl–2 or Bim can be produced at a level analogous to that produced by (a) or (b) above. It should be understood that any and all such equivalents are contemplated to fall within the scope of the present invention.

5. Targeting constructs

A gene disruption resulting in partial or complete loss of function in one or both alleles of *bcl–2* or *bim* is suitably effected using a targeting construct or vector. Any polynucleotide sequence capable of disrupting an endogenous gene of interest (*e.g.*, by introducing a premature stop codon, causing a frameshift mutation, disrupting proper splicing, etc.) may be employed in this regard. In a preferred embodiment, the vector, or an ancillary vector, comprises a positive selectable marker gene (*e.g.*, *hyg* or *neo*). The disruption may reduce or prevent the expression of *bcl–2* or *bim* or may render the resulting Bcl–2 or Bim completely non-functional. Reduced levels of Bcl–2 or Bim refer to a level of Bcl–2 or Bim which is lower than that found in a wild-type animal. The level of Bcl–2 or Bim produced in an animal of interest may be determined by a variety of methods including Western blot analysis of protein extracted from suitable cell types including, but not restricted to, kidney cells, lymphocytes or melanocytes. A lack of ability to produce functional Bcl–2 or Bim includes within its scope the production of undetectable levels of functional Bcl–2 or Bim (e.g., by Western blot analysis). In contrast, a functional Bcl–2 or Bim is a molecule which retains the biological activity of the wild-type Bcl–2 or Bim and which preferably is of the same molecular weight as the wild-type molecule.

Targeting vectors for homologous recombination will comprise at least a portion of the *bcl–2* or *bim* gene with the desired genetic modification, and will include regions of homology to the target locus. Those regions may be non-isogenic, but are preferably isogenic, to the target locus. Conveniently, markers for positive and negative selection are included. Methods for generating cells having targeted gene modifications through homologous recombination are known in the art. Various techniques for transfecting animal and particularly mammalian cells are described for example by Keown *et al.* (1990, *Methods in Enzymology* **185**: 527-537).

In a preferred embodiment, the targeting vector includes polynucleotide sequences comprising a selectable marker gene flanked on either side by *bcl–2* or *bim* gene sequences. The targeting vector will generally contain gene sequences sufficient to permit the homologous recombination of the targeting vector into at least one allele of the endogenous gene resident in the chromosomes of the target or recipient cell (*e.g.*, ES cells). Typically, the targeting vector will contain approximately 1 to 15 kb of DNA homologous to the endogenous *bcl–2* or *bim* gene (more than 15 kb or less than 5 kb of the endogenous *bcl–2* or *bim* gene sequences may be employed so long as the amount employed is sufficient to permit homologous recombination into the endogenous gene); this 1 to 15 kb of DNA is preferably divided on each side of the selectable marker gene.

The targeting construct may contain more than one selectable marker gene. The selectable marker is preferably a polynucleotide which encodes an enzymatic activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. Selectable markers may be "positive"; positive selectable markers typically are dominant selectable markers, *i.e.*, genes which encode an enzymatic activity which can be detected in any animal, preferably mammalian, cell or cell line (including ES cells). Examples of dominant selectable markers include the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as the *neo* gene) which confers resistance to the drug G418 in animal cells, the bacterial hygromycin G phosphotransferase (*hyg*) gene which confers resistance to the antibiotic hygromycin and the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the *gpt* gene) which confers the ability to grow in the presence of mycophenolic acid. Selectable markers may be 'negative'; negative selectable markers encode an enzymatic activity whose expression is cytotoxic to the cell when grown in an appropriate selective medium. For example, the HSV-tk gene is commonly used as a negative selectable marker. Expression of the HSV-tk gene in cells grown in the presence of gancyclovir or acyclovir is cytotoxic; thus, growth of cells in selective medium containing gancyclovir or acyclovir selects against cells capable of expressing a functional HSV TK enzyme.

When more than one selectable marker gene is employed, the targeting vector preferably contains a positive selectable marker (*e.g.*, the *neo* gene) and a negative selectable marker (*e.g.*, the Herpes simplex virus tk (HSV-tk) gene). The presence of the positive selectable marker permits the selection of recipient cells containing an integrated copy of the targeting vector whether this integration occurred at the target site or at a

random site. The presence of the negative selectable marker permits the identification of recipient cells containing the targeting vector at the targeted site (*i.e.*, which has integrated by virtue of homologous recombination into the target site); cells which survive when grown in medium which selects against the expression of the negative selectable marker do not contain a copy of the negative selectable marker.

Preferred targeting vectors of the present invention are of the "replacement-type", wherein integration of a replacement-type vector results in the insertion of a selectable marker into the target gene. Replacement-type targeting vectors may be employed to disrupt a gene resulting in the generation of a null allele (*i.e.*, an allele incapable of expressing a functional protein; null alleles may be generated by deleting a portion of the coding region, deleting the entire gene, introducing an insertion and/or a frameshift mutation, etc.) or may be used to introduce a modification (*e.g.*, one or more point mutations) into a gene.

6. Methods of producing the genetically modified animals of the invention

The genetically modified animals of the present invention are preferably generated by introduction of the targeting vectors into embryonal stem (ES) cells. ES cells are obtained by culturing pre-implantation embryos *in vitro* under appropriate conditions (Evans, *et al.*, 1981, *Nature* **292**: 154-156; Bradley, *et al.*, 1984, *Nature* **309**: 255-258; Gossler, *et al.*, 1986, *Proc. Natl. Acad. Sci. USA* **83**: 9065-9069; and Robertson, *et al.*, 1986, *Nature* **322**: 445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection using a variety of methods known to the art including electroporation, calcium phosphate co-precipitation, protoplast or spheroplast fusion, lipofection and DEAE-dextran-mediated transfection. Transgenes may also be introduced into ES cells by retrovirus-mediated transduction or by micro-injection. Cells are subsequently plated onto a feeder layer in an appropriate medium and those containing the transgene may be detected by employing a selective medium. Alternatively, PCR may be used to screen for ES cells which have integrated the transgene. After sufficient time for colonies to grow, they are picked and analysed for the occurrence of homologous recombination or integration of the vector. This PCR technique obviates the need for growth of the transfected ES cells under appropriate selective conditions prior to transfer into the blastocoel. Those colonies that are positive may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 week old superovulated females.

The ES cells are trypsinised, and the modified cells are injected into the blastocoel of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the vector. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected. For a review, see Jaenisch (1988, *Science* **240**: 1468-1474). The chimeric progeny are screened for the presence of the transgene and males and females having the transgene are mated to produce homozygous progeny. If the gene alterations cause lethality at some point in development, tissues or organs can be maintained as allogeneic or congenic grafts or transplants, or in *in vitro* culture.

Alternative methods for the generation of genetically modified animals are known to those skilled in the art. For example, embryonal cells at various developmental stages can be used to introduce transgenes for the production of genetically modified animals. Different methods are used depending on the stage of development of the embryonal cell. The zygote, particularly at the pronucleal stage (*i.e.*, prior to fusion of the male and female pronuclei), is a preferred target for micro-injection. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host genome before the first cleavage (Brinster, *et al.*, 1985, *Proc. Natl. Acad. Sci. USA* **82**: 4438-4442). As a consequence, all cells of the genetically modified non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbour the transgene. U.S. Pat. No. 4,873,191 describes a method for the micro-injection of zygotes.

Retroviral infection can also be used to introduce transgenes into a non-human animal. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Janenich, 1976, *Proc. Natl. Acad. Sci. USA* **73**: 1260-1264). Retroviral infection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, *et al.*, 1987, *EMBO J.* **6**: 383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoel (Jahner, D. *et al.*, 1982, *Nature* **298**: 623-628). It is also possible to introduce transgenes into the germline, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner, D. *et al.*, 1982, *supra*). An additional means of using

retroviruses or retroviral vectors to create genetically modified animals known to the art involves the micro-injection of retroviral particles or mitomycin C-treated cells producing retrovirus into the perivitelline space of fertilised eggs or early embryos (PCT International Application Publication No. WO 90/08832) and Haskell and Bowen, 1995, *Mol. Reprod. Dev.* 40: 386).

In selecting lines of an animal species to work the present invention, they may be selected for criteria such as embryo yield, pronuclear visibility in the embryos, reproductive fitness, colour selection of genetically modified offspring or availability of ES cell clones. For example, if genetically modified mice are to be produced, lines such as C57BL/6 may be preferred.

The age of the animals that are used to obtain embryos and to serve as surrogate hosts is a function of the species used. When mice are used, for example, pre-puberal females are preferred as they yield more embryos and respond better to hormone injections. In this regard, administration of hormones or other chemical compounds may be necessary to prepare the female for egg production, mating and/or implantation of embryos.

Genetically modified offspring of a surrogate host may be screened for the presence of the transgene by any suitable method. Screening may be accomplished by Southern or northern analysis using a probe that is complementary to at least a portion of the transgene or by PCR using primers complementary to portions of the transgene. Western blot analysis using an antibody against the protein encoded by the transgene may be employed as an alternative or additional method for screening. Alternative or additional methods for evaluating the presence of the transgene include without limitation suitable biochemical assays such as enzyme and/or immunological assays, histological stains for particular markers or enzyme activities and the like.

Progeny of the genetically modified mammals may be obtained by mating the genetically modified animal with a suitable partner or by *in vitro* fertilisation using eggs and/or sperm obtained from the genetically modified animal. Where *in vitro* fertilisation is used, the fertilised embryo is implanted into a surrogate host or incubated *in vitro* or both. Where mating is used to produce genetically modified progeny, the genetically modified animal may be back-crossed to a parental line, otherwise inbred or cross-bred with animals possessing other desirable genetic characteristics. In a preferred embodiment, such other

gene encoding a Bim-interacting pro-survival Bcl–2 family member including, but not limited to, Bcl–xL, Mcl–1, A–1 and Bcl–w. The progeny may be evaluated for the presence of the transgene using methods described above, or other appropriate methods.

Genetically modified animals comprising genetic alterations resulting in partial or complete loss of function in one or both alleles of *bcl–2* or *bim* find a number of uses. As discussed in more detail below, *bcl–2/bim* knockout mice provide a means for screening test compounds beneficial for modulating Bim and/or Bcl–2 function. In addition, these animals provide a means for screening compounds for the treatment or prevention in patients of conditions associated with the activation or inactivation of apoptosis.

7. ***Drug Screening Assays***

Through use of the subject transgenic animals or cells derived therefrom, one can identify ligands that directly or indirectly modulate, antagonise or agonise Bim and/or Bcl–2. Advantageously, the genetically modified animals of the invention provide convenient phenotypic screening for such ligands. Screening to determine drugs that lack effect on Bim and/or Bcl–2 is also of interest. Thus, the invention contemplates a method for screening a candidate agent for the ability to modulate apoptosis or cell survival. The method comprises administering a candidate agent to a genetically modified non-human animal as broadly described above which is associated with at least one predetermined first phenotype and detecting a change in phenotype. If the detected phenotype corresponds to at least one predetermined second phenotype associated with a different level and/or functional activity of Bim (a pro-apoptotic agent) or Bcl–2 (a pro-survival agent) or other Bim-interacting pro-survival Bcl–2 family member, than the level and/or functional activity of the pro-apoptotic or pro-survival agent associated with the or each predetermined first phenotype, then this indicates that the candidate agent is a modulator of apoptosis or cell survival.

A predetermined first phenotype preferably correlates with a different copy number of a gene selected from an endogenous gene that codes for a pro-apoptotic agent or for pro-survival agent mentioned above, than the copy number of that gene which correlates with a second predetermined phenotype.

The predetermined first and second phenotypes are preferably selected from the following:

(1) a substantially complete loss of function in a single allele of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene (*i.e.*, *bim*$^{+/-}$ *bcl–2*$^{-/-}$), which is characterised *inter alia* by amelioration of lymphopenia relative to the lymphopenia exhibited by *bcl–2*$^{-/-}$ animals;

(2) a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene (*i.e.*, *bcl–2*$^{-/-}$ *bim*$^{+/+}$), which is characterised *inter alia* by at least one phenotype selected from growth retardation, smaller ears, atrophic thymus and spleen, greying of hair and polycystic kidney disease;

(3) a substantially complete loss of function in both alleles of the endogenous *bim* gene (*i.e.*, *bim*$^{-/-}$ *bcl–2*$^{+/+}$), which is characterised *inter alia* by at least one phenotype selected from a 2–5-fold elevation in blood leukocytes, lymphadenopathy and SLE–like autoimmune glomerulonephritis; and

(4) a substantially complete loss of function in both alleles of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene (*i.e.*, *bim*$^{-/-}$ *bcl–2*$^{-/-}$), which is characterised *inter alia* by at least one phenotype selected from a robust lymphoid system and reduced greying of hair relative to that in *bcl–2*$^{-/-}$ animals.

Since the lymphoid system of *bcl–2*$^{-/-}$ *bim*$^{-/-}$ mice was found to be only slightly attenuated from that of *bim*$^{-/-}$ mice, the inventors concluded that the action of Bim must be mediated by at least one other pro-survival Bcl–2 family member such as, but not limited to, Bcl–xL, Mcl–1, A–1 and Bcl–w. Accordingly, *bcl–2*$^{-/-}$ *bim*$^{-/-}$ animals of the present invention might also find utility in screening for modulators of such other pro-survival agents. Thus, in another embodiment, the predetermined phenotype is associated with a substantially complete loss of function in both alleles of the endogenous *bim* gene, a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene and a substantially complete loss of function in one or both alleles of an endogenous gene encoding another Bim-interacting pro-survival Bcl–2 family member and is characterised *inter alia* by an even more attenuated lymphoid system than that observed in *bcl–2*$^{-/-}$ *bim*$^{-/-}$ animals. Suitably, the other Bcl–2 family member is selected from Bcl–xL, Mcl–1, A–1 and Bcl–w. Preferably, the other Bcl–2 family member is Bcl–xL.

The above predetermined phenotypes can be utilised advantageously to screen for agonists or antagonists of Bim or Bcl–2 or other Bim-interacting pro-survival Bcl–2 family member. For example, agonists of Bim will test positive in a *bcl–2*$^{-/-}$ *bim*$^{+/-}$ background if

they can effect the predetermined phenotype associated with the inactivation of *bcl–2* in the absence of *bim* disruption (*i.e.*, *bcl–2*$^{-/-}$ *bim*$^{+/+}$; growth retardation, smaller ears, atrophic thymus and spleen, greying of hair, polycystic kidney disease etc). Alternatively, antagonists of Bim will test positive in the same background if they can produce the predetermined phenotype associated with animals that are homozygous null for both *bim* and *bcl–2* (*i.e.*, *bcl–2*$^{-/-}$ *bim*$^{-/-}$; reduced greying of hair, robust lymphoid system etc). In another example, agonists of Bcl–2 will test positive in a *bcl–2*$^{+/-}$ *bim*$^{+/-}$ background if they can bring about the predetermined phenotype associated with *bcl–2*$^{+/+}$ *bim*$^{-/-}$ animals (*e.g.*, 2–5-fold elevation in blood leukocytes, lymphadenopathy, SLE–like autoimmune glomerulonephritis etc), whilst antagonists of Bcl–2 will test positive in the same background if they can lead to the predetermined phenotype associated with *bcl–2*$^{-/-}$ *bim*$^{+/-}$ animals (*e.g.*, amelioration of lymphopenia). Further, antagonists of the other Bim-interacting pro-survival Bcl–2 family member will test positive if they can lead to an even more attenuated lymphoid system than that observed in *bcl–2*$^{-/-}$ *bim*$^{-/-}$ animals.

The invention also encompasses the modulatory agents obtained or identified using the animals of the present invention.

8. *Uses of the modulatory agents of the invention*

The agents obtained or identified by the methods broadly described in Section 3 or Section 5 *supra* are useful for modulating the activation or inactivation of apoptosis in various cell types including the developing kidney, lymphocytes, melanocytes and neural cells and more particularly for treating or preventing conditions associated with the activation or inactivation of apoptosis associated with degenerative disorders, including disorders characterised by inappropriate cell proliferation or inappropriate cell death or in some cases, both. By way of example, agonists of Bim or antagonists of Bcl–2 are useful for treatment or prophylaxis of degenerative disorders characterised by inappropriate cell proliferation such as cancer and deletion of autoreactive lymphocytes in autoimmune disease, as for example described in Section 2. On the other hand, antagonists of Bim or agonists of Bcl–2 may be useful for treating or preventing degenerative disorders characterised by inappropriate cell death such as in chemotherapy or during HIV/AIDS or other viral infections, ischaemia or myocardial infarction, as for example described in Section 2.

The modulatory agent can be administered to a patient either by itself, or in pharmaceutical compositions where it is mixed with suitable pharmaceutically acceptable carrier. Depending on the specific conditions being treated, modulatory agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition. Suitable routes may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines.

The agents of the invention can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated in dosage forms such as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. These carriers may be selected from sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulphate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. The dose of agent administered to a patient should be sufficient to effect a beneficial response in the patient over time such as a reduction in the symptoms associated with the condition. The quantity of the agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof. In this regard, precise amounts of the agent(s) for administration will depend on the judgement of the practitioner. In determining the effective amount of the agent to be administered in the treatment or prophylaxis of the condition, the physician may evaluate the cellular level or functional activity of Bcl–2 or Bim and/or progression of

the condition. In any event, those of skill in the art may readily determine suitable dosages of the agents of the invention.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilisers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatine, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilising processes.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterise different combinations of active compound doses.

Pharmaceutical which can be used orally include push-fit capsules made of gelatine, as well as soft, sealed capsules made of gelatine and a plasticiser, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilisers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilisers may be added.

Dosage forms of the modulatory agents of the invention may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of an agent of the invention may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Modulatory agents of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulphuric, acetic, lactic, tartaric, malic, succinic, *etc.* Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

For any modulatory agent, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 as determined in cell culture (*e.g.*, the concentration of a test agent, which achieves a half-maximal inhibition of Bcl–2 or Bim activity). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of such agents can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit large therapeutic indices are preferred. The data obtained from these cell culture

The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilised. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See for example Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

Dosage amount and interval can be evaluated by routine methods in the art and may be adjusted individually to provide plasma levels of the active agent which are sufficient to maintain Bcl–2– or Bim–modulatory effects.

Alternately, one may administer the compound in a local rather than systemic manner, for example, *via* injection of the compound directly into a tissue often in a depot or sustained release formulation. Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tissue-specific antibody. The liposomes will be targeted to and taken up selectively by the tissue.

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non–limiting examples.

EXAMPLES

EXAMPLE 1

<u>*Generation of double knockout mice*</u>

Because *bcl–2*$^{-/-}$ mice on a mixed genetic background have a somewhat variable phenotype (Veis *et al.*; 1993; Nakayama *et al.*, 1993; Kamada *et al.*, 1995; Sorenson *et al.*, 1995; Michaelidis *et al.*, 1996), the inventors elected to study only those backcrossed to C57BL/6 animals for at least eight generations. On that background the phenotype was more homogenous and more uniformly severe than that described previously. All twenty *bcl–2*$^{-/-}$ mice produced by the present inventors were runts with short ears (Figure 1a), and all succumbed before 8 weeks of life to the fatal polycystic kidney disease. To assess how the *bcl–2* and *bim* genes interact *in vivo*, mice bearing a mutant *bcl–2* allele were bred with *bim*$^{-/-}$ animals, also backcrossed onto a C57BL/6 background. By two rounds of breeding, the inventors were able to generate *bcl-2*$^{-/-}$ mice lacking one or both alleles of *bim*.

Methods

bcl–2$^{+/-}$ mice (Nakayama *et al.*, 1994) were backcrossed onto the C57BL/6 background for more than ten generations. *bim*$^{-/-}$ mice of the 266Del strain (Bouillet *et al.*, 1998) backcrossed onto C57BL/6 background for eight generations or more were used for matings. Offspring were genotyped by PCR as described, and the mice that were *bcl–2*$^{+/-}$ *bim*$^{+/-}$ were bred with *bim*$^{-/-}$ animals to generate *bcl–2*$^{+/-}$ *bim*$^{-/-}$animals. These latter mice have been interbred to generate double homozygotes. *bcl–2*$^{-/-}$ *bim*$^{+/-}$ animals were obtained by crossing *bcl–2*$^{-/-}$ *bim*$^{-/-}$ animals with *bcl–2*$^{+/-}$ *bim*$^{+/-}$ or *bcl–2*$^{+/-}$ *bim*$^{+/+}$ animals.

bcl–2$^{-/-}$ mice were obtained mostly by interbreeding heterozygotes. Some *bcl–2*$^{-/-}$ *bim*$^{+/+}$ animals were also obtained from the breedings to produce double mutants. The phenotype of the *bcl–2*$^{-/-}$ mice was essentially the same as described previously by Veis *et al.* (1993) and Nakayama *et al.* (1993).

The wild–type *bcl–2* allele was identified using sense primer 5'–ATTCGTTCTCTTTATACTACCAAGG–3' [SEQ ID NO: 9] and anti–sense primer 5'–TGCTAAAGCGCATGCTCCAGACTG–3' [SEQ ID NO: 10]. The mutant *bcl–2* allele was identified using sense primer 5'–GACCCAATCTGGAAACCCTCCTGAT–3' [SEQ ID NO: 11] and anti–sense primer 5'–ATGTATGTACTTCATCACGAT–3' [SEQ ID NO:

12]. The wild–type *bim* allele was identified using sense primer 5'–CTGTCTGATGGACTGTGATCA–3' [SEQ ID NO: 13] and anti–sense primer 5'–CCTCCTTGTGTAAGTTTCGTT–3' [SEQ ID NO: 14]. To detect the *bim* null allele, sense primer 5'-ATGTATGTACTTCATCACGAT–3' [SEQ ID NO: 15] and anti–sense primer 5'–CTCAGTCCATTCATCAACAG–3' [SEQ ID NO: 16] were used.

EXAMPLE 2

Loss of a single bim allele in bcl–2$^{-/-}$ mice suffices to restore normal growth and ear length and to prevent kidney disease.

Surprisingly, the *bcl–2*$^{-/-}$ *bim*$^{-/-}$ and even the *bcl–2*$^{-/-}$ *bim*$^{+/-}$ offspring developed into healthy adults with ears of normal length (Figure 1b) and normal body weight (Figure 1c). To date, more than 40 *bcl–2*$^{-/-}$ *bim*$^{-/-}$and more than 25 *bcl–2*$^{-/-}$ *bim*$^{+/-}$ mice have been produced, none of which have died prematurely. Many of them are now more than six months old and all still look perfectly normal and healthy. Moreover, the kidneys of adult *bcl–2*$^{-/-}$ *bim*$^{-/-}$ and even *bcl–2*$^{-/-}$ *bim*$^{+/-}$ mice displayed a normal histology, with no signs of cysts (compare Figure 2a, 2c). Removal of even a single allele of *bim* thus appears to be sufficient to prevent completely the appearance of both polycystic kidney disease and growth retardation in *bcl–2*$^{-/-}$ mice. The concomitant rescue of these two defects suggests that the retarded growth is most probably a consequence of the renal dysfunction.

To investigate when kidney abnormality ensues in *bcl–2*$^{-/-}$ mice, the inventors compared the histology of kidneys from wt and *bcl–2*$^{-/-}$ mice from E12.5 to birth. As reported previously (Sorenson *et al.*, 1995), the neonatal *bcl–2*$^{-/-}$ kidney was much smaller than those from *bcl–2*$^{+/-}$ or wt littermates, with much fewer nephrons and smaller nephrogenic zones. Indeed, the inventors found that the metanephroi were already distinctly smaller than normal by E15.5, due mainly to a reduced mesenchymal area of the cortex (compare Figures 2c, d). The deficit in size of the *bcl–2*$^{-/-}$ kidney became increasingly pronounced later in gestation, and by birth cysts were already evident.

To clarify how Bim might influence kidney development, the inventors exploited mice engineered to have a β–galactosidase gene in the *bim* locus as a sensitive reporter for its expression (see Example 1). The reporter revealed intense *bim* expression in the mesenchymal tissue of the metanephric cap at E13.5 (Figure 2e) and E14.5 (not shown). Pertinently, in the *bcl–2*$^{-/-}$ kidney, apoptotic cells are especially abundant at this stage

within the mesenchymal regions of the metanephroi (Sorenson *et al.*, 1995; Nagata *et al.*, 1996). Thus, Bim is strongly expressed in the regions where cells are first lost from the *bcl–2*$^{-/-}$ kidney. Together with the finding that loss of even a single *bim* allele completely restores normal kidney development in *bcl–2*$^{-/-}$ mice, these results establish that Bim is responsible for the excessive apoptosis in the developing kidneys of mice lacking Bcl–2.

Methods

bim–β–gal mice were generated by homologous recombination in ES cells, using the same strategy as for the *bim* 266Del mice. The targeting construct removes the three first coding exons of the *bim* gene (exons 2, 3, and 4; Bouillet *et al.*, 2001) and replaces them with the β–gal cDNA and a lox–PGKneo–lox cassette. The ATG start codon of β–gal coincides exactly with the *bim* ATG initiation codon and no sequence other than that of exons 2, 3, 4 has been removed. Bim–β–gal mice have been crossed with Cre–expressing deleter mice (Schwenk *et al.*, 1995) to eliminate the PGK–neo cassette. The wild–type *bim* allele was identified using sense primer 5'–CCTTCTGATGTAAGTTCTGAG–3' [SEQ ID NO: 17] and anti–sense primer 5'–CATTGCACTGAGATAGTGGTT–3' [SEQ ID NO: 18]. Mutated allele was detected using a β–gal–specific sense primer 5'–AGGCACATGGCTGAATATCGAC–3' [SEQ ID NO: 19] and an antisense primer 5'–AACCAACTGTACCTTGGCTATA–3' [SEQ ID NO: 20] located in *bim* intron 2. This mutation also constitutes a null mutation for *bim*, as no Bim protein can be made from the remaining exons.

EXAMPLE 3

<u>Greying is prevented by loss of both bim alleles</u>

During the second hair follicle cycle at 5 to 7 weeks of age, *bcl-2*$^{-/-}$ mice (on a mixed C57BL/6x129 genetic background) develop a grey coat age (Veis *et al.*, 1993; Nakayama *et al.*, 1994; Kamada *et al.*, 1995). The *bcl-2*$^{-/-}$*bim*$^{+/-}$ mice (C57BL/6) still turned grey, but removal of both *bim* alleles almost completely prevented the coat color change (Figure 1b). Although the greying of Bcl-2-deficient mice was originally ascribed to defective melanin synthesis (Veis *et al.*, 1993), the inventors' observations support the conclusion that it reflects the loss of melanocytes (Yamamura *et al.* 1996). All the newly grown hairs on *bcl-2*$^{-/-}$*bim*$^{+/-}$ mice lacked melanin, and no melanocytes were discernible in their hair follicles (Figure 3b), whereas pigmentation was strong and melanocytes plentiful

in *bcl-2*$^{-/-}$*bim*$^{-/-}$ animals (Figure 3a). The grey appearance of the *bcl-2*$^{-/-}$*bim*$^{+/-}$ coats reflects incomplete shedding of the original black hairs, as shown by the pure white re-growth on a shaved patch (Figure 3c). Most likely, the partially pigmented hairs originally described reflected more variable melanocyte death on the mixed genetic background.

β-galactosidase staining of skin sections from *bim β-gal* mice demonstrated *bim* expression in the outer root sheath of the hair follicles (Figure 3d), where the melanocyte stem cells reside (Paus & Cotsarelis, 1999). Active melanocytes normally die shortly after the hair growth phase and must be regenerated from stem cells for the next hair growth cycle. Thus, Bcl-2 is essential to maintain production of skin melanocytes, and in its absence melanocyte precursors are killed by Bim. Since some 10% of the hairs on *bcl-2*$^{-/-}$ *bim*$^{-/-}$ mice still turn white (Figure 1b), other pro-apoptotic family members presumably can contribute to their apoptosis.

EXAMPLE 4

<u>*In the absence of Bim, bcl–2$^{-/-}$ cells create a robust lymphoid system.*</u>

To investigate how Bim and Bcl–2 interact functionally in the haemopoietic compartment, the inventors enumerated lymphoid, myeloid and erythroid cells in the blood, spleen and lymph nodes. Due to the early death of *bcl-2*$^{-/-}$ mice, useful data for that genotype could be derived only from wt mice whose haemopoietic compartment had been reconstituted with *bcl-2*$^{-/-}$ fetal liver cells (Matsuzaki *et al.*, 1997), a source of haemopoietic stem cells. For the other genotypes tested, the effects seen in unmanipulated and reconstituted mice were equivalent.

The numbers of erythroid progenitors and mature red cells was not altered by the absence of either Bcl-2 or Bim (data not shown), but those of B and T lymphocytes and myeloid cells (macrophages plus granulocytes) were all strongly affected (Figure 4a). Whereas Bcl-2 deficiency produced a marked reduction in lymphocytes (~10-fold) and a somewhat lower depression in myeloid cells (~5-fold), the *bim*$^{-/-}$ mice had 2- to 4-fold more B, T and myeloid cells than wt mice (Bouillet *et al.*, 1999). Significantly, lack of both *bcl-2* and *bim* restored myeloid cells to wt levels and produced mature lymphocyte numbers well above normal, indeed near the levels in the *bim*$^{-/-}$ animals. Interestingly, the *bcl-2*$^{-/-}$*bim*$^{+/-}$ mice had B, T and myeloid cell numbers intermediate between those in

bcl-2$^{-/-}$ and wt mice. Thus, loss of a single *bim* allele ameliorated the deficits produced by the absence of Bcl-2 and loss of both *bim* alleles more than restored normal numbers.

The massive apoptosis in the thymus, spleen and lymph nodes observed in the *bcl–2*$^{-/-}$ mice on a C57BL/6 background and on the mixed background (Veis *et al.*, 1993; Nakayama *et al.* 1994; Kamada *et al.*, 1995) after the first few weeks of life may be a consequence of the stress imposed by the renal deficiency in these mice. To circumvent such an indirect effect, the inventors performed hematopoietic reconstitution experiments. Foetal liver cells from wt, *bcl–2*$^{-/-}$, *bim*$^{-/-}$ or *bcl–2*$^{-/-}$ *bim*$^{-/-}$ embryos (all Ly5.2+) were transferred into irradiated C57BL/6–Ly5.1 recipients. The composition of their lymphoid organs was analysed 10–12 weeks later, using antibodies to Ly5.1 and Ly5.2 to distinguish donor–derived from host–derived leukocytes (Table 1).

The thymus was reconstituted very well, irrespective of the genotype of the donor cells. Total thymocyte numbers did not differ significantly, and the vast majority of the cells were donor–derived. Interestingly, the distribution of the four major thymocyte sub–populations in the recipients of either *bim*$^{-/-}$ or *bcl–2*$^{-/-}$ *bim*$^{-/-}$ cells were the same as in unmanipulated animals of the same genotypes (data not shown). Hence, the defect in T cell development induced by loss of Bim is cell intrinsic. The animals reconstituted with *bcl-2*$^{-/-}$ cells retained a normal distribution of the four major thymic sub–populations, confirming previous reports that loss of Bcl–2 does not affect initial T cell development in the thymus (Matsuzaki *et al.*, 1997).

In contrast to the thymus, the cell populations in the secondary lymphoid organs were strongly affected by the genotype of the donor cells (Table 1). Deficiency of Bcl–2 alone markedly impaired the reconstitution of spleen and lymph nodes. Spleen cellularity was half that in mice reconstituted with wt cells, and only 40–60% of the cells were Ly5.2+, *i.e.* *bcl–2*$^{-/-}$. The inferior ability of *bcl–2*$^{-/-}$ cells to generate and maintain mature lymphocytes was even more striking in the lymph nodes. The nodes were a quarter the size of those in mice reconstituted with wt cells, and only 25–35% of the cells were of donor origin. In marked contrast, total cell numbers in the spleen and lymph nodes of mice reconstituted with either *bim*$^{-/-}$ or *bcl–2*$^{-/-}$ *bim*$^{-/-}$ cells were approximately 1.7 times higher than those in mice reconstituted with wt cells, and more than 90% of their cells were donor derived. Thus, Bim can regulate the survival of mature lymphocytes independently of Bcl-2.

Methods

Immunofluorescence staining, flow cytometric analysis, and cell sorting

Peripheral blood erythrocytes and leukocytes were enumerated using a ZM model Coulter counter and platelets were counted in a Sysmex NE8000 counter (TOA, Kobe, Japan). Leukocytes in suspensions from spleen, lymph nodes and thymus were stained with eosin or trypan blue and counted in a hemocytometer.

Suspensions of cells isolated from thymus, spleen, lymph nodes, bone marrow or blood were surface stained with leukocyte–marker specific monoclonal antibodies that had been purified on protein G–Sepharose and conjugated with fluorescein isothiocyanate (FITC), R–phycoerythrin (PE), Cy5, or biotin (Molecular Probes). The monoclonal antibodies used and their specificities are: RA3–6B2: anti–CD45R(B220); GK1.5: anti–CD4; YTS 169: anti–CD8; 5.1: anti–IgM; 11–26C: anti–IgD; S7: anti CD43; T24.31.2: anti–Thy–1; H57.5721: anti–TCRβ; MI/70: anti–Mac–1; RA3–8C5: anti–Gr–l; Ter–119: anti–erythroid cell surface marker. Ly5.1; Ly5.2. Bound biotinylated antibodies were detected with PE–streptavidin or TRICOLOR–streptavidin (Caltag). Viable (excluding propidium iodide, PI) $CD4^+8^+$ thymocytes and mature T and B cells were purified on a Moflo (Cytomation); $FACStar^+$ or a modified FACS II cell sorter (Becton–Dickinson).

EXAMPLE 5

Bcl–2 and Bim affect the cytotoxic responses of mature B and T cells.

To gauge how Bcl–2 and Bim affected the apoptotic response to different cytotoxic insults, the inventors compared the survival *in vitro* of mature resting B cells and $CD8^+$ or $CD4^+$ T cells from lymph nodes of mice of the different genotypes, or of equivalent cells purified from reconstituted animals. (Since cells of a given genotype from unmanipulated and reconstituted animals behaved the same, those results were pooled.) The cells were cultured without cytokine support ('no treatment') or cultured in the presence of the glucocorticoid hormone dexamethasone or the topoisomerase inhibitor etoposide (VP16) (Figure 5).

In the spontaneous death response of B lymphocytes (Figure 5a), the *bcl–2*$^{-/-}$ cells were far more sensitive than wt cells, whereas the *bim*$^{-/-}$ cells survived twice as well as their wt counterparts. Interestingly, the double knockout B cells proved to be essentially as

refractory as the *bim*$^{-/-}$ cells. This observation indicates that Bim is required for the enhanced apoptosis of *bcl–2*$^{-/-}$ B cells; if another pro–apoptotic family member could compensate for the loss of Bim, the *bcl–2*$^{-/-}$ *bim*$^{-/-}$ cells would have remained highly sensitive to spontaneous death. The striking effect of *bim* dosage on survival during factor deprivation is illustrated by the results with *bcl–2*$^{-/-}$ cells bearing two, one or no *bim* alleles.

In the spontaneous T–cell death (Figure 5b), again absence of Bcl–2 rendered the cells far more sensitive, whereas absence of Bim was protective. The extent of death in cells lacking both genes, however, was comparable to that in wt cells. Results with CD8+ T cells are shown in Figure 4, but the CD4+ T cells behaved very similarly. Hence, in contrast to the B cells, Bcl–2 must be required for the killing action of Bim in both types of mature T cells.

In the response to etoposide (Figure 5c), as well as dexamethasone (data not shown), the mature B cells and both types of mature T cells behaved similarly. The absence of Bcl–2 rendered the cells far more sensitive to the glucocorticoid, whereas the absence of Bim offered substantial (5–fold) protection over wt cells. The *bcl–2*$^{-/-}$ *bim*$^{-/-}$ cells were essentially as sensitive as wt cells. Hence, the killing action of Bim in this response must be mediated largely through Bcl–2. Nevertheless, the observation that the presence of Bcl–2 enhanced survival in the absence of Bim probably indicates that one or more other pro–apoptotic family members contributes to the apoptotic response to glucocorticoids.

The inventors also examined the survival of CD4$^+$8$^+$ thymocytes sorted from *bim*$^{-/-}$ and the double knockout mice on culture in the presence of ionomycin, dexamethasone, PMA, etoposide, Fas ligand, or in the absence of added cytokines. The double knockout cells responded to these apoptotic stimuli in the same manner as *bcl–2*$^{+/+}$ *bim*$^{-/-}$ cells. For example, both were dramatically resistant to cytokine withdrawal and ionomycin compared to wt cells but as sensitive to PMA and Fas ligand. These results were not unexpected, as most CD4$^+$8$^+$ thymocytes contain very little if any Bcl–2 (Veis *et al.*, 1993) and *bcl–2*$^{-/-}$ thymocytes develop normally in reconstituted animals for at least two months (Table 1 and Matsuzaki *et al.*, 1997). Thus, Bim clearly can promote cell death independently of Bcl–2, presumably through countering other pro–survival family like Bcl–xL, because Bcl–xL is expressed much more highly than Bcl–2 in the CD4$^+$CD8$^+$ thymocytes and is required for their survival (Ma *et al.*, 1995).

Methods

Cell survival assays

Purified lymphocytes were cultured in the high glucose version of Dulbecco's modified Eagle's medium (DMEM) supplemented with 250 μM L–Asparagine, 50 pM 2–mercaptoethanol and 10% foetal calf serum (Biosciences). Cells were left untreated or were exposed to 0.01–1 pM dexamethasone (Sigma), 0.01–1 pg/mL ionomycin (Sigma), 0.2–20 ng/mL phorbol myristyl acetate (PMA; Sigma), 0.1–10 pg/mL etoposide (VP16; Della West) or recombinant human FLAG epitope–tagged FasL (1–100 ng/mL cross linked with 1 μg/mL anti–FLAG antibody, Alexis). Cell viability was quantified at daily intervals by staining with PI (5 μg/mL) plus FITC–labelled Annexin V and flow cytometric analysis in a FACScan (Becton Dickinson).

EXAMPLE 6

Bcl–2 is necessary for the survival signal conferred to mature T cells by IL–7

The survival signals from a number of cytokines act at least in part through the Bcl–2 family (Strasser *et al.*, 2000). In particular, IL–7 confers a survival signal to mature T cells (Maraskovsky *et al.*,1996) that is associated with the induction of bcl–2 expression (Maraskovsky *et al.*, 1997; Akashi *et al.*, 1997; von Freeden–Jeffry *et al.*, 1997). To assess whether Bcl–2 or Bim were important in the IL–7 response, the inventors monitored the survival of mature T cells deficient in either or both proteins in the presence of IL–7 (Figure 6). As expected, essentially all the wt and *bim*$^{-/-}$ T cells were alive after six days. By contrast, about 60% of *bcl–2*$^{-/-}$ *bim*$^{-/-}$ T cells had died, and the death toll was more than 90% for the *bcl–2*$^{-/-}$ *bim*$^{+/-}$ cells, and 100% for *bcl–2*$^{-/-}$ cells.

These results demonstrate that Bcl–2 is required for the survival signal conveyed by IL–7, even in the absence of Bim. They also show that the higher the dose of *bim* in the *bcl–2*$^{-/-}$ cells, the quicker they die. However, for a given dose of Bim, Bcl–2–expressing cells respond to IL–7, whereas *bcl–2*$^{-/-}$ cells are almost completely unresponsive. Thus, IL–7 conveys a survival signal that needs Bcl–2 to be fully integrated by the cell. In the absence of Bcl–2, the IL–7 signal cannot counteract other pro–apoptotic signals conveyed by Bim and other pro–apoptotic proteins.

EXAMPLE 7

Balance of BH3-only and Bcl-2-like proteins in the control of apoptosis and homeostasis

The striking effects of Bim dosage on kidney and external ear development and haematopoiesis in Bcl-2-deficient mice argue that Bim levels set the threshold for initiation of apoptosis in these diverse cell types. Since no evidence has emerged that loss of a single allele of *bcl-2* (or *bcl*-x, *bcl*-w, or *mcl-1*) increases cell death, the level of the pro-survival family members seems less limiting, perhaps because only a proportion of pro-survival molecules needs to be ligated by BH3-only proteins to induce apoptosis.

Not wishing to be bound by any one theory or mode of operation, the present inventors propose a model in which, for a given cell population, the extent of apoptosis is largely determined by the ratio of activated BH3-only protein molecules to the complement of pro-survival family members. In haematopoietic cells, for example, loss of Bcl-2 would tip the balance towards more apoptosis, but concomitant loss of one *bim* allele would then reduce cell death , and loss of both *bim* alleles would tip the balance to abnormally high cell survival.

The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

BIBLIOGRAPHY

Adams, J. M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Adams, J. M., and Cory, S. (2001). Life-or-death decisions by the Bcl-2 protein family. Trends Biochem. Sci. *26*, 61-66.

Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL. (1997). Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell *89*, 1033-1041.

Antonsson, B., Montessuit, S., Sanchez, B., and Martinou, J. C. (2001). Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J. Biol. Chem. *276*, 11615-11623.

Boletta, A., Qian, F., Onuchic, L. F., Bhunia, A. K., Phakdeekitcharoen, B., Hanaoka, K., Guggino, W., Monaco, L., and Germino, G. G. (2000). Polycystin-1, the gene product of *PKD1*, induces resistance to apoptosis and spontaneous tubulogenesis in MDCK cells. Mol. Cell *6*, 1267-1273.

Bouillet, P., Metcalf, D., Huang, D. C. S., Tarlinton, D. M., Kay, T. W. H., Köntgen, F., Adams, J. M., and Strasser, A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science *286*, 1735-1738.

Bouillet, P., Zhang, L. C., Huang, D. C., Webb, G. C., Bottema, C. D., Shore, P., Eyre, H. J., Sutherland, G. R., and Adams, J. M. (2001). Gene structure alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim. Mammal. Genome *12*, 163-168.

Coles, H. S., Burne, J. F., and Raff, M. C. (1993). Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. Development *118*, 777-784.

Conradt, B., and Horvitz, H. R. (1998). The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519-529.

Grantham, J. J., and Calvet, J. P. (2001). Polycystic kidney disease: In danger of being X-rated? Proc. Natl. Acad. Sci. U S A *98*, 790-792.

Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999). Bcl-2 family members and the mitochondria in apoptosis. Genes Dev. *13*, 1899-1911.

Hausmann, G., O'Reilly, L. A., van Driel, R., Beaumont, J. G., Strasser, A., Adams, J. M., and Huang, D. C. S. (2000). Pro-apoptotic apoptosis protease-activating Factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 or Bcl-x_L. J. Cell Biol. *149*, 623-634.

Horvitz, H. R. (1999). Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*. Cancer Res. *59*, 1701s-1706s.

Huang, D. C. S., and Strasser, A. (2000). BH3-only proteins – essential initiators of apoptotic cell death. Cell *103*, 839-842.

Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., Kitamura, Y., Kondoh, H., and Tsujimoto, Y. (1995). *bcl-2* deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus andspleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res. *55*, 354-359.

Knudson, C. M., and Korsmeyer, S. J. (1997). Bcl-2 and Bax function independently to regulate cell death. Nat. Genet. *16*, 358-363.

Knudson, C. M., Tung, K. S. K., Tourtellotte, W. G., Brown, G. A. J., and Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science *270*, 96-99.

Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellec, K., Philippe, M., and Roberts, J. M. (1991). Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. Cell *66*, 1217-28.

Koseki, C., Herzlinger, D., and al-Awqati, Q. (1992). Apoptosis in metanephric development. J. Cell Biol. *119*, 1327-1333.

Lindsten, T., Ross, A. J., King, A., Zong, W., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J.,

MacGregor, G. R., and Thompson, C. B. (2000). The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. Mol. Cell *6*, 1389-1399.

Lu, W., Peissel, B., Babakhanlou, H., Pavlova, A., Geng, L., Fan, X., Larson, C., Brent, G., and Zhou, J. (1997). Perinatal lethality with kidney and pancreas defects in mice with a targetted *Pkd1* mutation. Nat. Genet. *17*, 179-181.

Ma, A., Pena, J. C., Chang, B., Margosian, E., Davidson, L., Alt, F. W., and Thompson, C. B. (1995). Bclx regulates the survival of double-positive thymocytes. Proc. Natl. Acad. Sci. U S A *92*, 4763-7.

Maraskovsky E, Teepe M, Morrissey PJ, Braddy S, Miller RE, Lynch DH, Peschon JJ. (1996). Impaired survival and proliferation in IL-7 receptor-deficient peripheral T cells. J Immunol *157*, 5315-5323.

Maraskovsky E, O'Reilly LA, Teepe M, Corcoran LM, Peschon JJ, Strasser A. (1997). Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice. Cell *89*, 1011-1019.

Matsuzaki, Y., Nakayama, K.-I., Nakayama, K., Tomita, T., Isoda, M., Loh, D. Y., and Nakauchi, H. (1997). Role of bcl-2 in the development of lymphoid cells from the hematopoietic stem cell. Blood *89*, 853-862.

Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A., and Saikumar, P. (2001). Bcl-2 prevents bax oligomerization in the mitochondrial outer membrane. J. Biol. Chem. *20*, 20.

Moser, M., Pscherer, A., Roth, C., Becker, J., Mücher, G., Zerres, K., Dixkens, C., Weis, J., Guay-Woodford, L., Buettner, R., and Fässler, R. (1997). Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2β. Genes Dev. *11*, 1938-1948.

Murcia, N. S., Sweeney, W. E., Jr., and Avner, E. D. (1999). New insights into the molecular pathophysiology of polycystic kidney disease. Kidney Int. *55*, 1187-1197.

Murphy, K. M., Ranganathan, V., Farnsworth, M. L., Kavallaris, M., and Lock, R. B. (2000). Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. Cell Death Differ. 7, 102-111.

Nagata, M., Nakauchi, H., Nakayama, K.-I., Nakayama, K., Loh, D., and Watanabe, T. (1996). Apoptosis during an early stage of nephrogenesis induces renal hypoplasia in *bcl-2*-deficient mice. Am. J. Path. *148*, 1601-1611.

Nakayama, K., Nakayama, K.-I., Negishi, I., Kuida, K., Sawa, H., and Loh, D. Y. (1994). Targeted disruption of bcl-2αβ in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. U S A *91*, 3700-3704.

O'Connor, L., Strasser, A., O'Reilly, L. A., Hausmann, G., Adams, J. M., Cory, S., and Huang, D. C. S. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J. *17*, 384-395.

O'Reilly, L. A., Cullen, L., Visvader, J., Lindeman, G., Print, C., Bath, M. L., Huang, D. C. S., and Strasser, A. (2000). The pro-apoptotic BH3-only protein Bim is expressed in hemopoietic, epithelial, neuronal and germ cells. Am. J. Path. *157*, 449-461.

Ogilvy, S., Metcalf, D., Print, C. G., Bath, M. L., Harris, A. W., and Adams, J. M. (1999). Constitutive bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. Proc. Natl. Acad. Sci. U S A *96*, 14943-14948.

Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609-619.

Paus, R., and Cotsarelis, G. (1999). The biology of hair follicles. New Engl. J. Med. *341*, 491-497.

Putcha, G. V., Moulder, K. L., Golden, J. P., Bouillet, P., Adams, J. M., Strasser, A., and Johnson, E. M. J. (2001). Induction of Bim, a proapoptotic BH3-only Bcl-2 family member, is critical for neuronal apoptosis. Neuron *29*, 615-628.

Puthalakath, H., Huang, D. C. S., O'Reilly, L. A., King, S. M., and Strasser, A. (1999). The pro-apoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell *3*, 287-296.

Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S.

W. (1997). Structure of Bcl-x_L-Bak peptide complex: recognition between regulators of apoptosis. Science *275*, 983-986.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A *cre*-transgenic mouse strain for the ubiquitous deletion of *loxP*-flanked gene segments including deletion in germ cells. Nucleic Acids Res. *23*, 5080-5081.

Shinjyo, T., Kuribara, R., Inukai, T., Hosoi, H., Kinoshita, T., Miyajima, A., Houghton, P. J., Look, A. T., Ozawa, K., and Inaba, T. (2001). Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors. Mol. Cell. Biol. *21*, 854-864.

Sorenson, C. M., Rogers, S. A., Korsmeyer, S. J., and Hammerman, M. R. (1995). Fulminant metanephric apoptosis and abnormal kidney development in bcl-2-deficient mice. Am. J. Physiol. *268*, F73-F81.

Strasser, A., O'Connor, L., and Dixit, V. M. (2000). Apoptosis signaling. Annu. Rev. Biochem. *69*, 217-245.

Trudel, M., Lanoix, J., Barisoni, L., Blouin, M.-J., Desforges, M., L'Italien, C., and D'Agati, V. (1997). C-myc-induced apoptosis in polycystic kidney disease is Bcl-2 and p53 independent. J. Exp. Med. *186*, 1873-1884.

Veis, D. J., Sorenson, C. M., Shutter, J. R., and Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229-240.

von Freeden-Jeffry, U., Solvason, N., Howard, M., and Murray, R. (1997). The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. Immunity *7*, 147-154.

Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727-730.

Whitfield, J., Neame, S. J., Paquet, L., Bernard, O., and Ham, J. (2001). Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron *29*, 629-643.

Yamamura, K., Kamada, S., Ito, S., Nakagawa, K., Ichihashi, M., and Tsujimoto, Y. (1996). Accelerated disappearance of melanocytes in *bcl-2*-deficient mice. Cancer Res. *56*, 3546-3550.

Zheng, T. S. (2001). Death by design: the big debut of small molecules. Nat. Cell Biol. *3*, E43-E46.

CLAIMS

1. A genetically modified non-human animal having a partial or complete loss of function in one or both alleles of the endogenous *bcl–2* gene and in one or both alleles of the endogenous *bim* gene.

2. A genetically modified animal according to claim 1, which comprises a disruption in at least one allele of the endogenous *bim* gene and in at least one allele of the endogenous *bcl–2* gene.

3. A genetically modified animal according to claim 2, wherein the disruption has been introduced into the genome of the animal by homologous recombination with a targeting construct in an embryonic stem cell such that the targeting construct is stably integrated in the genome of the animal, and wherein the disruption results in a reduced or abrogated level and/or functional activity of an expression product corresponding to the at least one allele.

4. A genetically modified animal according to claim 1, which has a substantially complete loss of function in a single allele of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

5. A genetically modified animal according to claim 4, which exhibits at least one of the following phenotypes: normal kidney development, weight gain and health and reduced lymphopenia, relative to the lymphopenia exhibited by an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene but having normal function in both alleles of the endogenous *bim* gene.

6. A genetically modified animal according to claim 1, which has a substantially complete loss of function in both alleles of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

7. A genetically modified animal according to claim 4, which exhibits at least one of the following phenotypes: a robust lymphoid system and reduced greying of hair relative to the greying of hair exhibited by an animal of the same species as the genetically modified animal, and having a substantially complete loss of function in both alleles of

the endogenous *bcl–2* gene but having normal function in both alleles of the endogenous *bim* gene.

8. A genetically modified animal according to claim 6, which further has a substantially complete loss of function in one or both alleles of an endogenous gene encoding another Bim-interacting pro-survival Bcl–2 family member.

9. A genetically modified animal according to claim 8, wherein the other Bim-interacting pro-survival Bcl–2 family member is selected from Bcl–xL, Mcl–1, A–1 and Bcl–w.

10. A genetically modified animal according to claim 8, wherein the other Bim-interacting pro-survival Bcl–2 family member is Bcl–xL.

11. A genetically modified animal according to claim 8, which exhibits an even more attenuated lymphoid system than the lymphoid system exhibited by an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene and in both alleles of the endogenous *bim* gene

12. A genetically modified animal according to claim 1, which is selected from the order Rodentia.

13. A genetically modified animal according to claim 1, which is a mouse.

14. A cell having a partial or complete loss of function in one or both alleles of the endogenous *bcl–2* gene and a partial or complete loss of function in one or both alleles of the endogenous *bim* gene.

15. A cell according to claim 14, which comprises a disruption in at least one allele of the endogenous *bim* gene and a disruption in at least one allele of the endogenous *bcl–2* gene.

16. A cell according to claim 15, wherein the disruption has been introduced into the genome of the cell by homologous recombination with a targeting construct in an embryonic stem cell such that the targeting construct is stably integrated in the genome of the cell, and wherein the disruption results in a reduced or abrogated level and/or functional activity of an expression product corresponding to the at least one allele.

17. A cell according to claim 14, which has a substantially complete loss of function in a single allele of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

18. A cell according to claim 14, which has a substantially complete loss of function in both alleles of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

19. A cell according to claim 18, which further has a substantially complete loss of function in one or both alleles of an endogenous gene encoding another Bim-interacting pro-survival Bcl–2 family member.

20. A cell according to claim 19, wherein the other Bim-interacting pro-survival Bcl–2 family member is selected from Bcl–xL, Mcl–1, A–1 and Bcl–w.

21. A cell according to claim 19, wherein the other Bim-interacting pro-survival Bcl–2 family member is Bcl–xL.

22. A cell derived from the cell of claim 14.

23. A method for screening a candidate agent for the ability to modulate apoptosis or cell survival, comprising:

(a) administering a candidate agent to a genetically modified non-human animal having a partial or complete loss of function in at least one allele of the endogenous *bcl–2* gene, which results in a reduced or abrogated level and/or functional activity of Bcl–2, and a partial or complete loss of function in at least one allele of the endogenous *bim* gene, which results in a reduced or abrogated level and/or functional activity of Bim, wherein the genetically modified animal has at least one predetermined first phenotype in the absence of administering the candidate agent to the animal, and

(b) detecting at least one predetermined second phenotype associated with a different level and/or functional activity of a molecule selected from Bim, Bcl–2 or other Bim-interacting pro-survival Bcl–2 family member, than the level and/or functional activity of the molecule associated with the predetermined first phenotype(s), which indicates that the candidate agent is a modulator of apoptosis or cell survival.

24. A method according to claim 23, wherein a predetermined first phenotype correlates with a different copy number of a gene selected from the endogenous *bcl*-2 gene and the endogenous *bim* gene, than the copy number of that gene which correlates with the predetermined second phenotype.

25. A method according to claim 23, wherein the predetermined first phenotype is associated with a substantially complete loss of function in a single allele of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

26. A method according to claim 25, wherein the predetermined first phenotype is characterised by amelioration of lymphopenia relative to the lymphopenia exhibited by an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene but having normal function in both alleles of the endogenous *bim* gene.

27. A method according to claim 23, wherein the predetermined first phenotype is associated with a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene and with normal function in both alleles of the endogenous *bim* gene.

28. A method according to claim 27, wherein the predetermined first phenotype is selected from growth retardation, atrophic thymus and spleen, greying of hair, polycystic kidney disease and smaller ears than those of an animal of the same species as the genetically modified animal and having normal function in both alleles of the endogenous *bcl-2* gene.

29. A method according to claim 23, wherein the predetermined first phenotype is associated with a substantially complete loss of function in both alleles of the endogenous *bim* gene and with normal function in both alleles of the endogenous *bcl-2* gene.

30. A method according to claim 29, wherein the predetermined first phenotype is selected from lymphadenopathy, SLE–like autoimmune glomerulonephritis and a 2–5-fold elevation in blood leukocytes relative to the number of blood leukocytes in an animal of the same species as the genetically modified animal and having normal function in both alleles of the endogenous *bim* gene.

31. A method according to claim 23, wherein the predetermined first phenotype is associated with a substantially complete loss of function in a single allele of the endogenous *bim* gene and in a single allele of the endogenous *bcl-2* gene.

32. A method according to claim 23, wherein the predetermined first phenotype is associated with a substantially complete loss of function in both alleles of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

33. A method according to claim 32, wherein the predetermined first phenotype is selected from a robust lymphoid system and reduced greying of hair relative to the greying of hair exhibited by an animal of the same species as the genetically modified animal and having normal function in both alleles of the endogenous *bcl–2* gene.

34. A method according to claim 23, wherein the other Bcl–2 family member is selected from Bcl–xL, Mcl–1, A–1 and Bcl–w.

35. A method according to claim 23, wherein the other Bcl–2 family member is Bcl–xL.

36. A method according to claim 25, wherein a candidate agent for enhancing apoptosis or reducing cell survival tests positive if it effects at least one predetermined second phenotype associated with an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene and normal function in both alleles of the endogenous *bim* gene.

37. A method according to claim 36, wherein the or each predetermined second phenotype is selected from growth retardation, atrophic thymus and spleen, greying of hair, polycystic kidney disease and smaller ears than those of an animal of the same species as the genetically modified animal and having normal function in both alleles of the endogenous *bcl-2* gene.

38. A method according to claim 25, wherein a candidate agent for reducing apoptosis or enhancing cell survival tests positive if it effects at least one predetermined second phenotype associated with an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene and in both alleles of the endogenous *bim* gene.

39. A method according to claim 38, wherein the or each predetermined second phenotype is selected from a robust lymphoid system and reduced greying of hair relative to the greying of hair exhibited by an animal of the same species as the genetically modified animal and having normal function in both alleles of the endogenous *bcl–2* gene.

40. A method according to claim 31, wherein a candidate agent for reducing apoptosis or enhancing cell survival tests positive if it effects at least one predetermined second phenotype associated with an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bim* gene and normal function in both alleles of the endogenous *bcl-2* gene.

41. A method according to claim 40, wherein the or each predetermined second phenotype is selected from lymphadenopathy, SLE–like autoimmune glomerulonephritis and a 2–5-fold elevation in blood leukocytes relative to the number of blood leukocytes in an animal of the same species as the genetically modified animal and having normal function in both alleles of the endogenous *bim* gene.

42. A method according to claim 31, wherein a candidate agent for enhancing apoptosis or reducing cell survival tests positive if it effects at least one predetermined second phenotype associated with an animal of the same species as the genetically modified animal and having a substantially complete loss of function in a single allele of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

43. A method according to claim 42, wherein the predetermined second phenotype is amelioration of lymphopenia relative to the lymphopenia exhibited by an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bcl–2* gene but having normal function in both alleles of the endogenous *bim* gene.

44. A method according to claim 32, wherein a candidate agent for reducing apoptosis or enhancing cell survival tests positive if it effects at least one predetermined second phenotype associated with an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bim* gene, in both alleles of the endogenous *bcl–2* gene and in one or both

alleles of the endogenous gene encoding the other Bim-interacting pro-survival Bcl–2 family member.

45. A method according to claim 42, wherein the predetermined second phenotype is an even more attenuated lymphoid system than the lymphoid system exhibited by an animal of the same species as the genetically modified animal and having a substantially complete loss of function in both alleles of the endogenous *bim* gene and in both alleles of the endogenous *bcl–2* gene.

46. A method for modulating the activation or inactivation of apoptosis in a cell, the method comprising administering to a patient in need of such treatment an agent, which has been identified by the method of claim 23, for a time and under conditions sufficient to modulate the activation or inactivation in the cell.

47. A method for treatment and/or prophylaxis in a patient of a condition associated with the activation or inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent, which has been identified by the method of claim 23, for a time and under conditions sufficient to treat or prevent the condition.

48. A method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation or inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent, which modulates the level and/or functional activity of Bim and which has been identified by the method of claim 23, for a time and under conditions sufficient to treat or prevent the disorder.

49. A method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation of apoptosis, the method comprising administering to the patient an effective amount of an agent, which reduces, abrogates or otherwise suppresses the level and/or functional activity of Bim and which has been identified by the method of claim 23, for a time and under conditions sufficient to treat or prevent the disorder.

50. A method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent, which increases, enhances or otherwise elevates the level and/or functional activity of Bim and which has been identified by

the method of claim 23, for a time and under conditions sufficient to treat or prevent the disorder.

51. A method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation or inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent which modulates the level and/or functional activity of a BH3–only protein for a time and under conditions sufficient to treat or prevent the disorder.

52. A method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the activation of apoptosis, the method comprising administering to the patient an effective amount of an agent which reduces, abrogates or otherwise suppresses the level and/or functional activity of a BH3–only protein for a time and under conditions sufficient to treat or prevent the disorder.

53. A method for the treatment and/or prophylaxis in a patient of a degenerative disorder associated with the inactivation of apoptosis, the method comprising administering to the patient an effective amount of an agent which increases, enhances or otherwise elevates the level and/or functional activity of a BH3–only protein for a time and under conditions sufficient to treat or prevent the disorder.

54. A method according to any one of claims 51 to 53, wherein the BH3–only protein is selected from BAD, BID, Hrk/DP5, Bik/Nbk, Blk, Bmf, NOXA, PUMA or Bim, or variants thereof.

55. A method according to any one of claims 51 to 53, wherein the BH3–only protein is Bim.

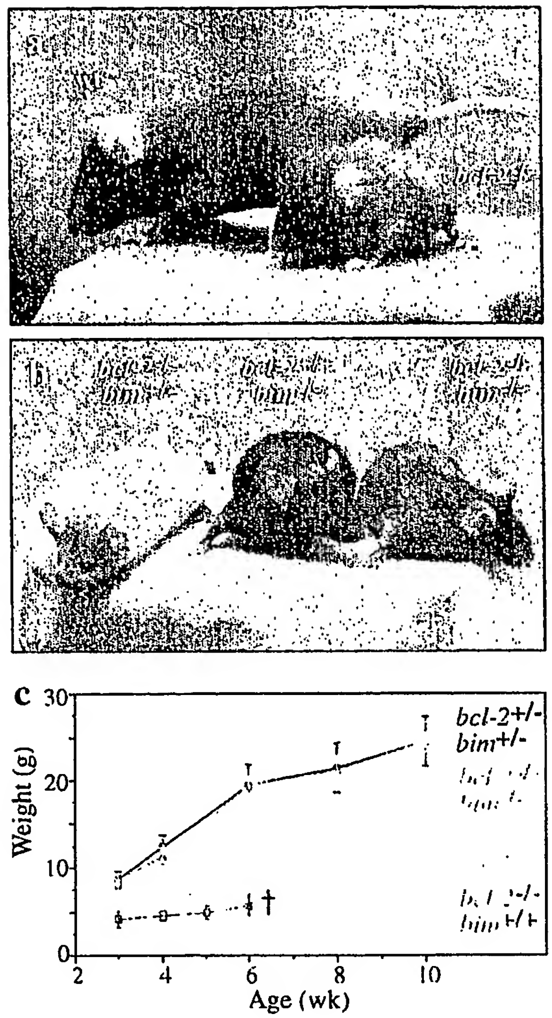

FIGURE 1

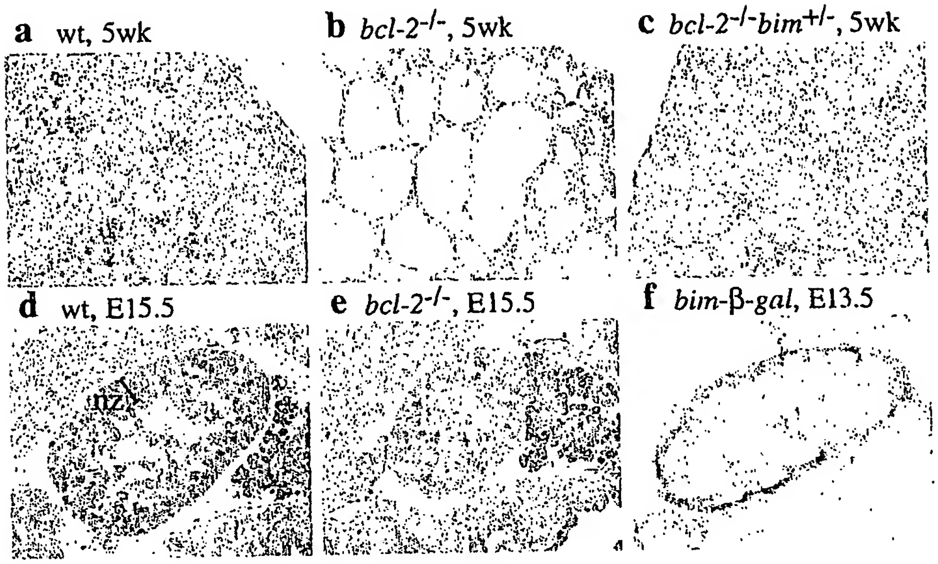
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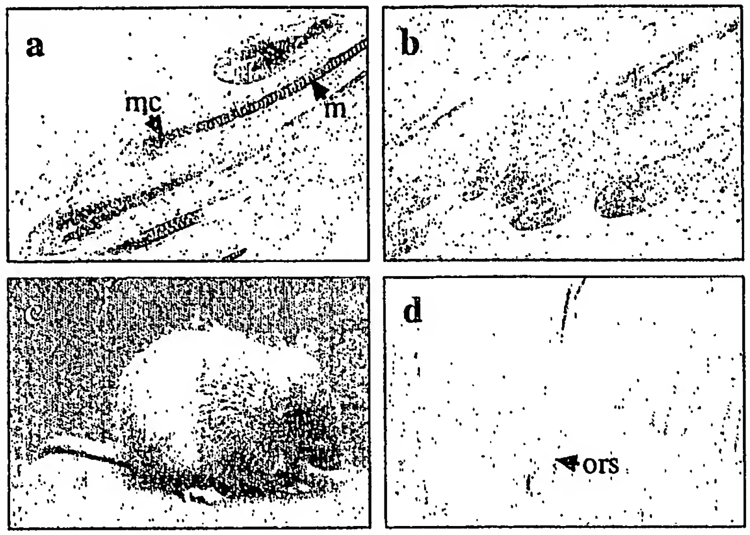
FIGURE 2



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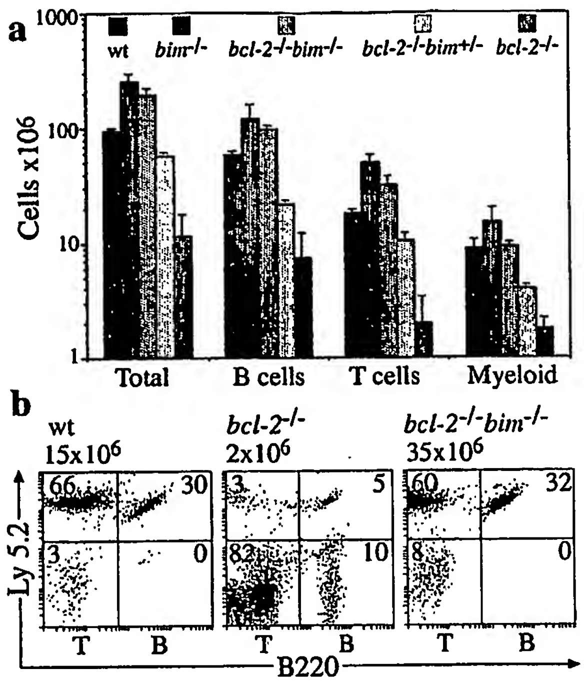
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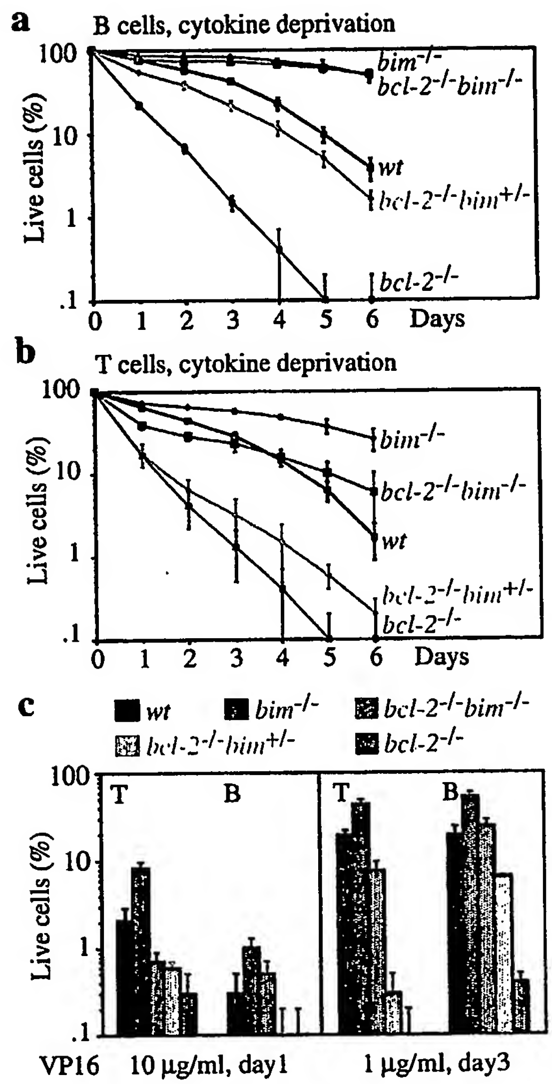
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FIGURE 5

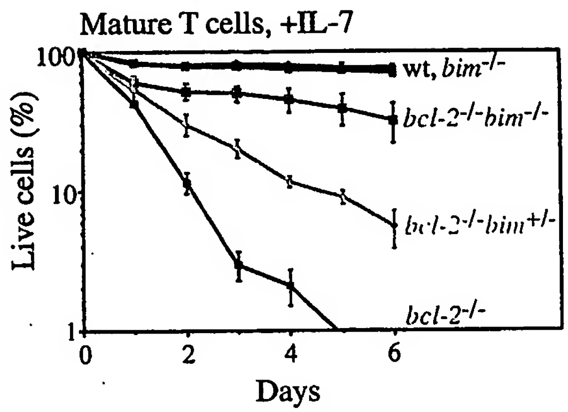

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FIGURE 6

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Zhang, Li-Chen
Adams, Jerry McKee
Bouillet, Philippe

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Pro Leu Gly Ala Ala Pro Thr Pro Gly Ile Phe Ser Phe Gln Pro Glu
    40                  45                  50

agc aac cca atg ccc gct gtg cac cgg gag atg gct gcc agg acg tct      1627
Ser Asn Pro Met Pro Ala Val His Arg Glu Met Ala Ala Arg Thr Ser
55                  60                  65                  70

cct ctc agg ccc ctc gtt gcc acc gct ggg cct gcg ctc agc cct gtg      1675
Pro Leu Arg Pro Leu Val Ala Thr Ala Gly Pro Ala Leu Ser Pro Val
                75                  80                  85

cca cca tgt gtc cat ctg acc ctc cgc cgg gct ggg gat gac ttc tct      1723
Pro Pro Cys Val His Leu Thr Leu Arg Arg Ala Gly Asp Asp Phe Ser
            90                  95                  100

cgt cgc tac cgt cgt gac ttc gca gag atg tcc agt cag ctg cac ctg      1771
Arg Arg Tyr Arg Arg Asp Phe Ala Glu Met Ser Ser Gln Leu His Leu
        105                 110                 115

acg ccc ttc acc gcg agg gga cgc ttt gcc acg gtg gtg gag gaa ctc      1819
Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala Thr Val Val Glu Glu Leu
    120                 125                 130

ttc agg gat ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc      1867
Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala Phe Phe Glu Phe
135                 140                 145                 150

ggt ggg gtc atg tgt gtg gag agc gtc aac agg gag atg tca ccc ctg      1915
Gly Gly Val Met Cys Val Glu Ser Val Asn Arg Glu Met Ser Pro Leu
                155                 160                 165

gtg gac aac atc gcc ctg tgg atg act gag tac ctg aac cgg cat ctg      1963
Val Asp Asn Ile Ala Leu Trp Met Thr Glu Tyr Leu Asn Arg His Leu
            170                 175                 180

cac acc tgg atc cag gat aac gga ggc tgg gtaggtgcat gtctggttga        2013
His Thr Trp Ile Gln Asp Asn Gly Gly Trp
        185                 190

atgagtctgg gctttgatct caaggccaag atgcgcaggt tggggtgtga gtggattctg    2073

ggtcaaaatg ggccattgag cagatgaaat aaagtcgagt ttgtagcttt gctcccctga    2133

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Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro Leu Phe Asp
        195                 200                 205

ttc tcc tgg ctg tct ctg aag acc ctg ctc agc ctg ccc tgg gtc ggg     3008
Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Pro Trp Val Gly
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gcc tgc atc act ctg ggt gca tac ctg ggc cac aag tgaggtcgac          3054
Ala Cys Ile Thr Leu Gly Ala Tyr Leu Gly His Lys
225                 230                 235

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Gly Asp Ala Asp Ala Ala Pro Leu Gly Ala Ala Pro Thr Pro Gly Ile
35 40 45

Phe Ser Phe Gln Pro Glu Ser Asn Pro Met Pro Ala Val His Arg Glu
50 55 60

Met Ala Ala Arg Thr Ser Pro Leu Arg Pro Leu Val Ala Thr Ala Gly
65 70 75 80

Pro Ala Leu Ser Pro Val Pro Pro Cys Val His Leu Thr Leu Arg Arg
85 90 95

Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala Glu Met
100 105 110

Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala
115 120 125

Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile
130 135 140

Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser Val Asn
145 150 155 160

Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met Thr Glu
165 170 175

Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp
180 185 190

Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro Leu Phe Asp
195 200 205

Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Pro Trp Val Gly
210 215 220

Ala Cys Ile Thr Leu Gly Ala Tyr Leu Gly His Lys
225 230 235

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Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
20 25 30

Gly Asp Ala Asp Ala Ala Pro Leu Gly Ala Ala Pro Thr Pro Gly Ile
35 40 45

Phe Ser Phe Gln Pro Glu Ser Asn Pro Met Pro Ala Val His Arg Glu
50 55 60

Met Ala Ala Arg Thr Ser Pro Leu Arg Pro Leu Val Ala Thr Ala Gly
65 70 75 80

Pro Ala Leu Ser Pro Val Pro Pro Cys Val His Leu Thr Leu Arg Arg
85 90 95

Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala Glu Met
100 105 110

Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala
115 120 125

Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile

130 135 140

Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser Val Asn
145 150 155 160

Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met Thr Glu
165 170 175

Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp
180 185 190

Val Gly Ala Cys Leu Val Glu
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ggtcaggaat tctaggaaag gacagtcaaa ggccagtgcc catccttgca ccaatctaat 300

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1               5                   10                  15

gga caa ttg cag cct gct gag agg cct ccc cag ctc agg cct ggg gcc       96
Gly Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30

cct acc tcc cta cag aca gaa ccg caa gac agg agc ccg gca ccc atg      144
Pro Thr Ser Leu Gln Thr Glu Pro Gln Asp Arg Ser Pro Ala Pro Met
        35                  40                  45

agt tgt gac aag tca aca caa acc cca agt cct cct tgc cag gcc ttc      192
Ser Cys Asp Lys Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe
    50                  55                  60

aac cac tat ctc agt gca atg gct tcc ata cga cag tct cag gag gaa      240
Asn His Tyr Leu Ser Ala Met Ala Ser Ile Arg Gln Ser Gln Glu Glu
65                  70                  75                  80

cct gaa gat ctg cgc ccg gag ata cgg att gca cag gag ctg cgg cgg      288
Pro Glu Asp Leu Arg Pro Glu Ile Arg Ile Ala Gln Glu Leu Arg Arg
                85                  90                  95

atc gga gac gag ttc aac gaa act tac aca agg agg gtg ttt gca aat      336
Ile Gly Asp Glu Phe Asn Glu Thr Tyr Thr Arg Arg Val Phe Ala Asn
            100                 105                 110

gat tac cgc gag gct gaa gac cac cct caa atg gtt atc tta caa ctg      384
Asp Tyr Arg Glu Ala Glu Asp His Pro Gln Met Val Ile Leu Gln Leu
        115                 120                 125

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Leu Arg Phe Ile Phe Arg Leu Val Trp Arg Arg His
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Pro Thr Ser Leu Gln Thr Glu Pro Gln Asp Arg Ser Pro Ala Pro Met
        35                  40                  45
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Ser Cys Asp Lys Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe
50 55 60

Asn His Tyr Leu Ser Ala Met Ala Ser Ile Arg Gln Ser Gln Glu Glu
65 70 75 80

Pro Glu Asp Leu Arg Pro Glu Ile Arg Ile Ala Gln Glu Leu Arg Arg
85 90 95

Ile Gly Asp Glu Phe Asn Glu Thr Tyr Thr Arg Arg Val Phe Ala Asn
100 105 110

Asp Tyr Arg Glu Ala Glu Asp His Pro Gln Met Val Ile Leu Gln Leu
115 120 125

Leu Arg Phe Ile Phe Arg Leu Val Trp Arg Arg His
130 135 140

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Gly Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
20 25 30

cct acc tcc cta cag aca gaa ccg caa ggt aat ccc gac ggc gaa ggg 144
Pro Thr Ser Leu Gln Thr Glu Pro Gln Gly Asn Pro Asp Gly Glu Gly
35 40 45

gac cgc tgc ccc cac ggc agc cct cag ggc ccg ctg gcc cca ccg gcc 192
Asp Arg Cys Pro His Gly Ser Pro Gln Gly Pro Leu Ala Pro Pro Ala
50 55 60

agc cct ggc cct ttt gct acc aga tcc cca ctt ttc atc ttt gtg aga 240
Ser Pro Gly Pro Phe Ala Thr Arg Ser Pro Leu Phe Ile Phe Val Arg
65 70 75 80

aga tct tct ctg ctg tcc cgg tcc tcc agt ggg tat ttc tct ttt gac 288
Arg Ser Ser Leu Leu Ser Arg Ser Ser Ser Gly Tyr Phe Ser Phe Asp
85 90 95

aca gac agg agc ccg gca ccc atg agt tgt gac aag tca aca caa acc 336

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Thr Asp Arg Ser Pro Ala Pro Met Ser Cys Asp Lys Ser Thr Gln Thr
            100                 105                 110

cca agt cct cct tgc cag gcc ttc aac cac tat ctc agt gca atg gct       384
Pro Ser Pro Pro Cys Gln Ala Phe Asn His Tyr Leu Ser Ala Met Ala
        115                 120                 125

tcc ata cga cag tct cag gag gaa cct gaa gat ctg cgc ccg gag ata       432
Ser Ile Arg Gln Ser Gln Glu Glu Pro Glu Asp Leu Arg Pro Glu Ile
    130                 135                 140

cgg att gca cag gag ctg cgg cgg atc gga gac gag ttc aac gaa act       480
Arg Ile Ala Gln Glu Leu Arg Arg Ile Gly Asp Glu Phe Asn Glu Thr
145                 150                 155                 160

tac aca agg agg gtg ttt gca aat gat tac cgc gag gct gaa gac cac       528
Tyr Thr Arg Arg Val Phe Ala Asn Asp Tyr Arg Glu Ala Glu Asp His
                165                 170                 175

cct caa atg gtt atc tta caa ctg tta cgc ttt atc ttc cgt ctg gta       576
Pro Gln Met Val Ile Leu Gln Leu Leu Arg Phe Ile Phe Arg Leu Val
            180                 185                 190

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Trp Arg Arg His
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<213>  Mus musculus

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            20                  25                  30


Pro Thr Ser Leu Gln Thr Glu Pro Gln Gly Asn Pro Asp Gly Glu Gly
        35                  40                  45


Asp Arg Cys Pro His Gly Ser Pro Gln Gly Pro Leu Ala Pro Pro Ala
    50                  55                  60


Ser Pro Gly Pro Phe Ala Thr Arg Ser Pro Leu Phe Ile Phe Val Arg
65                  70                  75                  80


Arg Ser Ser Leu Leu Ser Arg Ser Ser Ser Gly Tyr Phe Ser Phe Asp
                85                  90                  95


Thr Asp Arg Ser Pro Ala Pro Met Ser Cys Asp Lys Ser Thr Gln Thr
            100                 105                 110
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Pro Ser Pro Pro Cys Gln Ala Phe Asn His Tyr Leu Ser Ala Met Ala
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Ser Ile Arg Gln Ser Gln Glu Glu Pro Glu Asp Leu Arg Pro Glu Ile
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Arg Ile Ala Gln Glu Leu Arg Arg Ile Gly Asp Glu Phe Asn Glu Thr
145   150   155   160

Tyr Thr Arg Arg Val Phe Ala Asn Asp Tyr Arg Glu Ala Glu Asp His
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Pro Gln Met Val Ile Leu Gln Leu Leu Arg Phe Ile Phe Arg Leu Val
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Trp Arg Arg His
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<400> 10
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<210> 11
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<400> 11
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<223> Mutant bcl-2 antisense primer

<400> 12
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<210> 13
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<223> Wild-type bim sense primer

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<210> 20
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<220>
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INTERNATIONAL SEARCH REPORT	International application No. PCT/AU02/01325

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: A01K 67/02, C12N 5/06, G01N 33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, WIPIDS, MEDLINE, BIOSIS : bim, bcl-2, cell lines, animal model, knock out

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	BOUILLET P et al., " Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim ". DEVELOPMENTAL CELL, (2001 Nov) 1 (5) 645-53.	1-22
A	HUANG D C., " The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4". EMBO JOURNAL, (1998 Feb 16) 17 (4) 1029-39.	1-22
A	O'CONNOR L., " Bim : a novel member of the Bcl-2 family that promotes apoptosis". EMBO JOURNAL, (1998 Jan 15) 17 (2) 384-95.	1-22

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2002	**13 November 2002 (13.11.02)**
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**Christopher Luton** Telephone No : (02) 6283 2256

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU02/01325

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : 46-55

   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   The claims define the use of compounds identified using the applicant's invention for treatment of a condition associated with apoptosis. Such claims are not acceptable because they do not define compounds produced using the applicant's invention. Processes of detection, identification or screening do not produce products they simply provide further information about the properties of pre-existing compounds. Claiming a compound in terms of its inherent feature, even if these features have not previously been recognised, does not alter the fact that the claim is still a claim to the use of a compound *per se*.

3. ☐ Claims Nos :

   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01325

C (Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	O'REILLY L A et al., " The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells". AMERICAN JOURNAL OF PATHOLOGY, (2000 Aug) 157 (2) 449-61.	1-22
A	CHENG E H et al.," BCL-2 , BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis". MOLECULAR CELL, (2001 Sep) 8 (3) 705-11	1-22